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Rapid Development of Homozygous Transgenic Rice using Anther Culture Harboring Rice *chitinase* Gene for Enhanced Sheath Blight Resistance

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Abstract

Transgenic rice was developed from both calli and immature embryos of popular cultivar 'Swarna' with a rice *chitinase* gene (*chi11*) by particle gun bombardment. Homozygous dihaploid transgenics were produced in less than a year through the anther culture of primary transgenics. Stable integration and expression of the *chi11* transgene were confirmed by Southern and western analyses, respectively of primary as well as anther culture-derived dihaploid transgenics. The homozygous transgenics with functional transgene and varied levels of chitinase activity showed enhanced resistance to sheath blight fungus. The bioassay data were correlated with the molecular and biochemical results.

Introduction

Rice is the staple food for 3 billion people mostly from the developing countries and provides 40-70% of the calories in their diet. Several factors limit the fullest realization of yield potential of rice cultivars. Diseases have been the constant major biotic threat, lowering the productivity of superior rice cultivars. Sheath blight disease caused by the fungus *Rhizoctonia solani* Kühn, is considered globally as the second most important disease after blast but the widest spread disease in all the rice-growing countries (Dasgupta, 1991). It causes yield loss of 5.2% to 50 % in India (Mathur, 1983). This disease has so far eluded any concrete and inexpensive solution for poor rice farmers. Moreover, use of agrochemicals in controlling this disease has been ineffective, inefficient, and not environment-friendly. The most effective and desirable strategy is to incorporate disease resistance genes into commercially acceptable cultivars. So far, conventional breeding has not been very successful in spite of substantial efforts, mainly due to the lack of donor parents resistant to sheath blight in the available rice gene pool (Bonman *et al.,* 1992), although genes conferring only partial resistance have recently been identified (Pan *et al.,* 1999).

In response to the pathogen attack, a cascade of signaling events occur within the host-plant cell ultimately producing pathogenesis-related (PR) proteins that play key roles in the plant defense mechanism (Datta and Muthukrishnan, 1999). Hence, a genetic engineering strategy involving constitutive, high-level expression of single or combinations of PR proteins with different modes of action against target organisms may provide broad-spectrum, durable resistance to rice varieties (Datta *et al.,* 1999a). Among the PR protein genes, the most attractive candidate for manipulation of a single-gene defense approach is the gene encoding for an antifungal enzyme, chitinase, which hydrolyses the chitin oligomer, an integral and major component of the fungal cell wall. The *chitinase* gene has been transferred successfully through genetic engineering to rice (Lin *et al.,* 1995; Baisakh *et al.,* 1999; Nishizawa *et al.,* 1999; Datta *et al.,* 2000) and several other crop plants including tobacco, tomato, rape, sorghum, wheat etc. The transgenic plants were found to have enhanced resistance to sheath blight disease and the level of resistance was positively correlated with the amount of chitinase enzyme produced in the transgenics (Broglie *et al.,* 1991; Lin *et al.,* 1995; Datta *et al.,* 2000). The stability of the transgene expression in the subsequent generations depends upon the homozygosity at the transgenic locus, thus making the transgenic lines fixed. This could be achieved by

advancement of selfing generations at least up to T_2 (Tu *et al.*, 1998) which requires a minimum of 2 years or more depending on the photosensitive reaction of the genotype(s). However, doubled-haploid breeding through anther culture (AC) could be successfully employed to reduce this time period for attaining homozygosity, more particularly when the transgenes are integrated in more than one locus/site in the chromosome, a rare but unavoidable phenomenon in biolistic transformation (Baisakh *et al.*, 1999). However, care should be taken to avoid the gametoclonal variation, an undesirable feature in transgenic research.

We chose a widely adapted and adopted indica rice variety Swarna having a yield potential of 8.0 t ha^{-1} (Rao *et al.*, 1983). It is extensively grown in most of the rice-growing countries such as India, Bangladesh, and Myanmar, for its high grain quality and consumer appeal besides its moderate resistance to brown spot and field tolerance for bacterial blight. However, Swarna is highly susceptible to sheath blight disease, which causes considerable yield loss. We report here for the first time the rapid production of homozygous transgenic rice using anther culture of the primary transgenics with an agronomically important gene, *chitinase*, that confer resistance to sheath blight.

Materials and methods

Plasmid construct

The transformation vector pGL2CaMV35S-CHI11 (**Fig. 1**) (Lin *et al.*, 1995) was used in the experiment. The vector carries a 1.1-kb DNA fragment corresponding to rice *chitinase* gene encoding a class I chitinase enzyme (Huang *et al.*, 1991), and a 1.1-kb *hph* gene as selectable marker, both driven independently under the control of 35S constitutive promoter from CaMV, in the backbone of rice transformation vector pGL2 (Datta *et al.*, 1990).

Rice transformation

Sterilized immature embryos(IEs) of Swarna isolated from immature grains (10-12 days after pollination) and embryogenic calli(EC) developed from mature/immature embryos were used as the target explants for bombardment. The procedure for plant transformation till recovery of putative transformants were same as described earlier (Datta *et al.*, 1999b). The only modification was that hygromycin was used at 30 mgl^{-1} throughout the selection cycles instead of 50 mgl^{-1}. The primary transgenics and further seed progenies were grown to maturity in the transgenic greenhouse maintained at a day/night temperature regime of 29/23 ℃ .

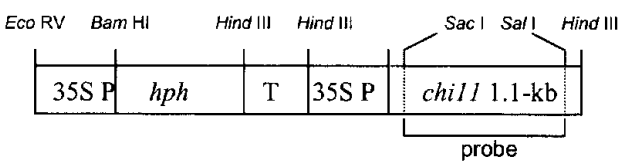

Fig. 1 Partial map of the rice transformation vector pGL2CaMV35S-CHI11

Genomic DNA isolation and Southern blot analysis

Genomic DNA was extracted from freshly collected or freeze-dried rice leaves as per Dellaporta *et al.* (1983). Ten-μg aliquots of total DNA were digested with the respective restriction enzymes (Gibco-BRL, Geithersburg, MD) as described in the figures for different purposes and electrophoresed on 1% (w/v) agarose gel. The DNA fragments were transferred to Hybond-N^+ nylon membrane (Amersham, Arlington Heights, IL) according to the manufacturer's instructions. The *Sac*I/*Sal*I digested 1.1-kb *chi11* and/or *Bam*HI/*Hin*dIII released 1.1-kb *hph* fragment of pGL2CaMV35S-CHI11 were labeled with (α-^{32}P) dCTP using the Rediprime Labeling Kit (Amersham, Arlington Heights, IL) and used as the hybridization probes.

Anther culture

Immature panicles still inside the boots were collected from the secondary and tertiary tillers of the Southern positive primary transgenics grown in the transgenic greenhouse and were given cold shock at 10 ℃ for 10 days. The boots were rinsed with absolute alcohol for 1 min followed by sterilization with 50% Clorox for 20 min. The spikelets were cut open and the anthers were plated onto petri dishes (60x15 mm^2) containing 10 ml of semisolid N_6 (Chu *et al.*, 1975) medium fortified with 2 mgl^{-1} 2,4-D, 0.5 mgl^{-1} kinetin, 30 gl^{-1} maltose, and 0.75% (w/v) agar ($N_6$2m). The anther cultures were incubated in the dark at 26-27 ℃ . After 1 month, pollen embryoids/calli from the responding anthers were transferred within 1 week of formation to MS (Murashige and Skoog, 1962) -based regeneration medium containing 0.5 mgl^{-1} NAA, 1.5 mgl^{-1} BAP, 0.5 mgl^{-1} kinetin and 30 gl^{-1} maltose solidified with 0.7% (w/v) agar as described earlier (Baisakh and Rao, Unpublished). The green plantlets were transferred to MS basal medium (MS_0) for rooting, and subsequent cultural operations were done in the same way as for the transgenic plants.

Protein extraction, HPT assay and Immunoblot Analysis

Fresh leaves (0.8-1.0 g) from transgenics, anther culture-derived plants as well as non-transformed control plants, were ground with liquid nitrogen and

extracts were made with 1.0-1.5 ml buffer [0.05 M Tris-HCl (pH 7.0) containing 10% (v/v) glycerol and 0.1 M PMSF] at 4 ℃ . The supernatants were collected by centrifuging at 13,000 rpm for 10 min followed by a second centrifugation for 5 min. The protein concentrations were determined using a modified bicinchonic acid (BCA) reagent (PIERCE, Rockford, IL) with bovine serum albumin (BSA) as the standard.

The HPT assay and the western blot analysis were performed following Datta *et al.* (1990) and Tu *et al.* (1998), respectively.

Chitinase activity assay

The activity of the chitinase in the transgenics and the non-transformed control plants was estimated according to Boller *et al.* (1992). Crude enzyme extract was made from the leaf tissues with 0.1M sodium citrate as the extraction buffer. Deacetylated chitosan was prepared to colloidal suspension and radiolabeled with 0.2 m*l* acetic anhydride containing 5 mCi [^{3}H] acetic anhydride, and was used as the substrate for the enzymatic reaction. The enzymatic reaction mixture containing 100 μl crude enzyme extract, 50 μl sodium citrate (pH 5.0), and 100 μl radioactive colloidal chitin was incubated at 37 ℃ for 10 min. To the mixture, 250 μl of trichloroacetic acid (TCA) was added and the supernatant was eluted after centrifugation at 13000 rpm for 10 min. The radioactivity count of the chitinase activity was taken by a scintillation counter with 4ml of scintillation liquid. The activity of chitinase releasing the molecules of soluble chito-oligosaccharides was determined from a standard curve using dilutions of a highly active sample and then extrapolating the activity to high dilution (Dr. Jean-Marc Neuhaus, Switzerland, personal communication).

Inoculum preparation and Bioassay

Preparation of inoculum of *Rhizoctonia solani* fungus and bioassay were done as reported earlier (Datta *et al.,* 1999b). The symptoms caused by the fungal infection were scored at weekly intervals as per Ou (1985) and the relative integrated infection index (RI) was calculated as described earlier (Datta *et al.,* 1999b).

Results

Rice transformation, HPT, and Southern blot analysis of primary transformants

A partial map of the transformation vector, pGL2(CaMV35S-CHI11), carrying *chi11* and *hph* (selectable marker) genes both driven under the control of the constitutive 35S promoter from CaMV is depicted in **Figure 1**. The *chitinase* was introduced into IEs and ECs of Swarna by the particle gun gene delivery system. A total of 7 primary transformants (given code no. SHC1 to SHC7) were obtained from three independently selected hygromycin-resistant calli from two independent transformation events (four plants, SHC1...SHC4, from two calli obtained from IEs and 3 plants, SHC5..SHC7, from one callus from ECs). Preliminary screening of the T_0 plants by the hygromycin phosphotransferase (HPT) assay revealed that all seven plants were positive for HPT activity. The plants were normal and self-fertile. Southern blot analysis of the 7 HPT-positive primary transgenics for *chi11* showed that all four plants derived from IEs had the same banding pattern, which was different from that found in the three plants obtained from ECs, which also had an identical banding pattern among them. The integration of the intact promoter and transgene was confirmed by the presence of the expected 1.5-kb *Hin*dIII fragment (35S/*chi11* fusion) in all the transgenics (**Fig. 2**). The independent status of the transgenics from two transformation experiments was also confirmed by a Southern analysis for the *hph* gene clearly showing distinct patterns of integration of *hph* (**Fig. 3**). As seen from the Southern blot, altogether there were four distinct patterns of integration of *hph* distributed over the seven plants obtained from three independently selected calli. The integration site analysis of the chitinase transgene in Swarna primary transgenics (**Fig. 4**) revealed a single-locus integration in all the transgenics except SHC5, which had only one hybridization signal higher than the endogenous chitinase common to non-trans-

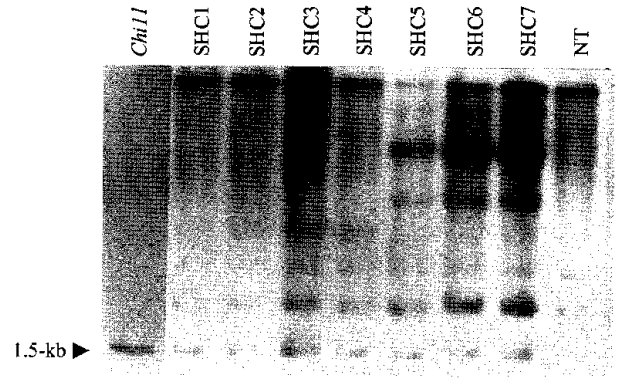

Fig. 2 Southern blot of primary transgenics of Swarna (SHC 1-7) showing the integration pattern of *chi11* gene
Genomic DNA was digested with *Hin*dIII , Southern blotted and hybridized with 1.1-kb *chi11* fragment (*Sac* I /*Sal* I) of pGL2*chi11* as the probe
NT=non-transformed; *chi11*=positive contorol (1.5-kb *Hin*dIII frament from pGL2*chi11*)

Table 1. Anther culture response of five primary transgenics (T_0) of rice cultivar Swarna

T_0 plant	Total no. of anthers plated	No. of anthers producing calli	No. of green plants produced
SHC1	280	24(8.6)*	8
SHC2	122	10(8.2)	6
SHC3	191	14(7.3)	6
SHC4	200	17(8.5)	7
SHC5	95	7(7.4)	4

* Numbers in the parentheses represent the percentage of anthers producing calli

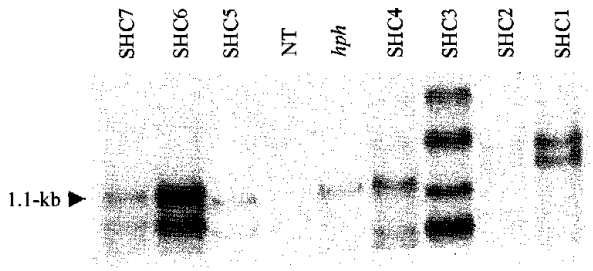

Fig. 3 Southern blot of primary transgenics of Swarna (SHC 1-7) showing difference in the integration pattern of *hph* gene
GEnomic DNA was digested with *Bam*HI/*Hin*dIII, Southern blotted and hybridized with 1.1-kb hph fragment (*Bam*HI/*Hin*dIII) of pGL2*chi11* as the probe
NT=non-transformed; *hph*=posiitive control

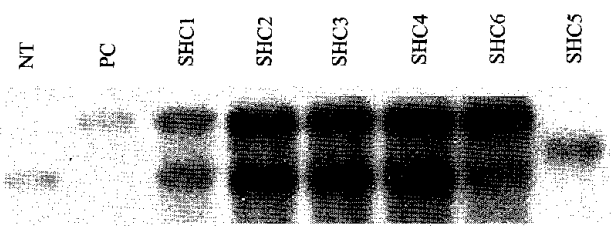

Fig. 4 Southern blot analysis of Swarna primary transgenics (SHC 1-6) showing differential intergration of *chi11* transgene.
NT=non-transformed control; PC=positive control (*Eco*RV fragment of pGL2*chi11*) Genomic DNA was digested with *Eco*RV and Southern probed with *chi11* fragment(*Sac* I /*Sal* I released fragment of pGL2*chi11*); NT=non-transformed control; positive contorol (*Eco*RV released fragment of pGL2*chi11*)

formed as well as other transgenics.

Anther culture of the T_0 transgenics, and HPT and Southern analysis of the anther culture-derived plants

Anther culture was done from 5 primary transgenics (SHC1 - SHC5) having enough tillers as described in the materials and methods section. We used the secondary and tertiary tillers to ensure the harvest of seeds from the primary/mother tiller for growing subsequent seed progenies. The anther calli

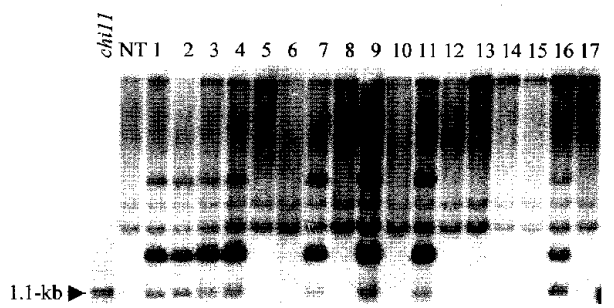

Fig. 5 Southern blot analysis of anther culture-deraived lines of Swarna (# 1 to 7) showing segregation of *chi11* transgene expected in the pollern plants.Genomic DNA was digested with *Sac* I /*Sal* I and Southern probed with *chi11* fragment (*Sac* I /*Sal* I released fragment of pGL2*chi11*); NT=non-transformed contorol; *chi11*=positive contorol(same as the probe)

were observed after 3-4 weeks of dark incubation. Plantlets were regenerated from the anther calli/embryoids (one week old) within 2-3 weeks after transfer to regeneration medium. The transgenic lines showed almost the same levels of response to anther culture except for SHC3 and SHC5, which had less induction frequency (**Table 1**). A total of 31 green plants (given codes SHC1AC1, SHC1AC2, etc) were obtained from anther culture of five different T_0 plants (**Table 1**). Rooting on MS basal medium, transfer to culture solution and subsequently to soil were accomplished in another four weeks. The plants flowered in a period of ~2 months after transfer to soil, and another one month for maturity. Thus it took about 6-7 months after obtaining the primary transgenics (which took 5 months), i.e. a total of about a year to obtain homozygous transgenics from the start of transformation process. AC-derived plants were also initially screened for HPT activity. Eight AC-derived plants out of the 20 plants analyzed were positive for *hph* expression (data not shown). The HPT-positive AC lines were Southern-analyzed for *chi11* and the presence of the intact 1.1-kb *Sac*I / *Sal*I fragment confirmed the stable integration of the *chitinase* gene (**Fig. 5**). As expected, the banding patterns of the AC lines were

the same as those of T_0 parental lines (data not shown).

Immunoblot analysis of the T_0 and AC lines

The HPT-positive and Southern-positive T_0 and AC lines were analyzed for expression of the *chi11* integrated in the genome by immunoblot analysis. Western blot showed the presence of a 35-kDa protein in the primary transgenics as well as AC lines expected for *chi11* transgene expression, which was absent in the non-transformed control plant (**Figs. 6 & 7**). A 28-kDa size protein was observed invariably in all the transgenics as well as the non-transformed control plant showing the expression of the endogenous chitinase genes present in the rice genome. Apart from these two sizes of proteins, another 30-kDa protein band, a proteolytic degradation product of 35-kDa protein (Datta *et al.*, 2000), with varying intensity was detected in all the transgenic plants analyzed.

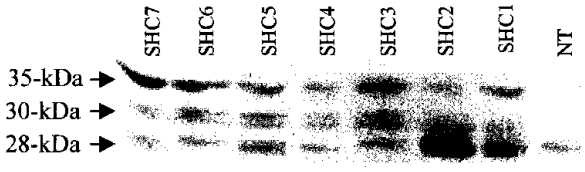

Fig. 6 Immunoblot analysis expressing *chi11* transgene-specific 35-kDa protein in the primary transgenics of Swarna(SHC1-7) which is absent the non-transformed control(NT)

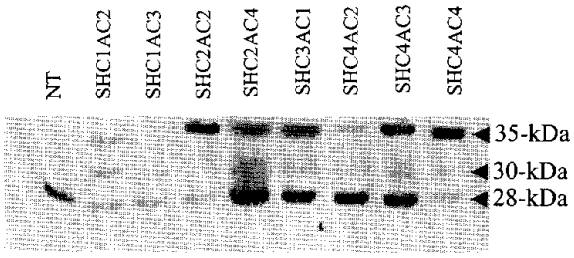

Fig. 7 Immunblot analysis showing the stable expression of the 35-kDa chitinase protein inthe anther culture-derived transgenics of Swarna; NT=non-transformed

Chitinase activity and bioassay of the AC- derived transgenics

The transgenic AC lines showed variation in their chitinase activity (**Fig. 8**). The two doubled haploids (SHC1AC7 and SHC4AC2) showed higher activity than the control, whereas the line SHC1AC2, a doubled haploid, and other haploids had lower values of released chito-oligosaccharides. The homozygosity of a transgenic dihaploid (SHC1AC7) was further confirmed from the western blot analysis by the expression of 35-kDa protein in all of its progenies (data not shown).

From the bioassay results based on the relative integrated infection index (**Fig. 9**), it was clear that the transgenic AC lines exhibited lower intensity of disease symptom than the non-transformed control

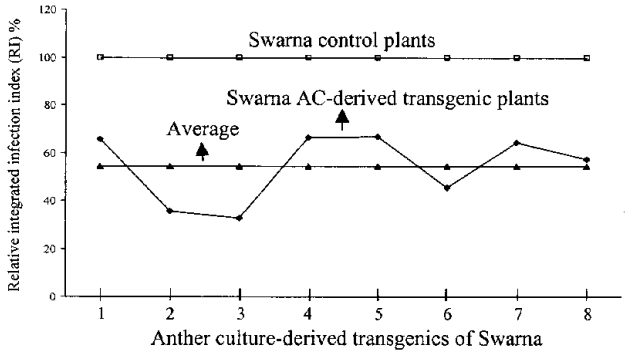

Fig. 9 Sheeath blight bioassay of transgenic anther culture (AC) - derived lines of Swarna

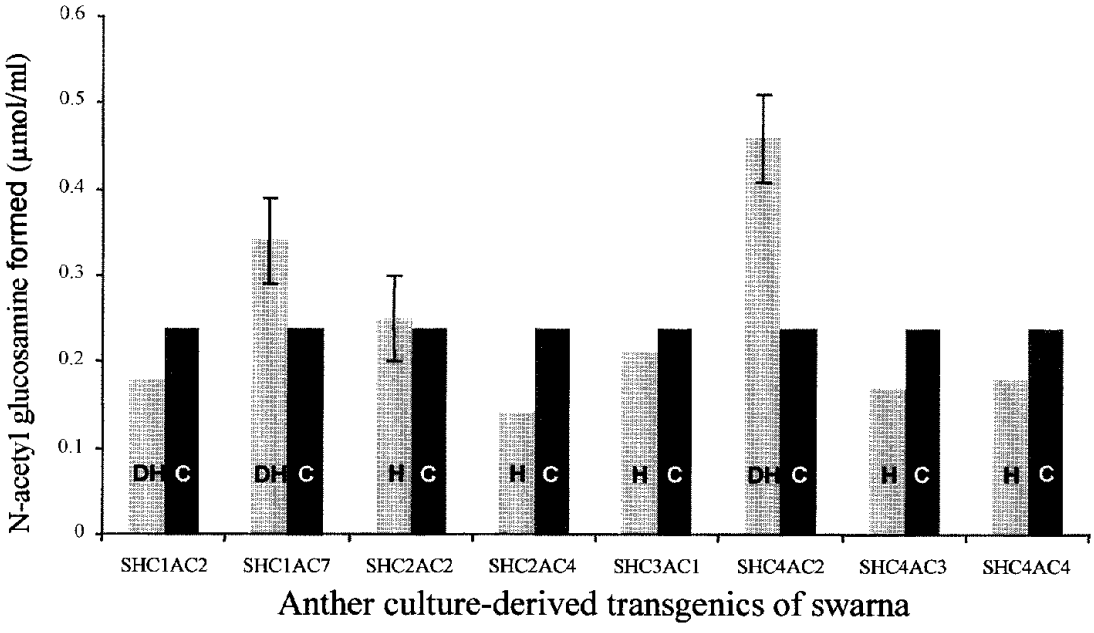

Fig. 8 Chitinase activity of transgenic anther culture (AC) -derived lines of Swarna DH=doubled haploid; H=haploid; C=non transformed control

plants, in which the disease severity was quite high, even progressing to or spreading over the whole leaf and panicles in the later stages. On an average, the AC lines exhibited ~55% enhancement in resistance to sheath blight fungus.

Discussion

We have introduced the class I PR-gene *chitinase* into a commercially important cultivar, Swarna, through the biolistic method of transformation. The low number of transgenics obtained in our experiment was due to the higher level of sensitivity of this particular genotype to selection, although Swarna has been shown to be a very responsive variety with normal tissue culture. From the control experiments, we found that 30 mgl^{-1} of hygromycin B is sufficient to kill the non-transformed IEs/EC (data not shown). All genotypes do not respond equally in AC as evident from our earlier work with another variety, Tulasi. This might be due to the loss of the green plant regenerability of the anther calli after a long period of tissue culture under selection. However, efforts are underway to optimize the selection pressure and the time under selection to minimize the albinism problem so that we can obtain the homozygous fixed lines of the proven transgenics in a single tissue culture cycle. Three independent primary transgenics were obtained with a distinct pattern of integration as evident from the Southern analysis. The plants SHC1 and SHC2 did not show the 1.1-kb band expected for *hph*, which could presumably be due to deletion or duplication of the *hph* coding sequence during or after the integration leading to rearrangements. However, other transgenics had at least one copy of the intact *hph*. The AC lines positive for *hph* were also Southern-positive for *chitinase*, indicating stable co-integration and co-segregation of both the transgenes. The expression of HPT and chitinase enzymes in the transgenics as well as the AC lines confirms the stable integration and expression of both the genes without any changes during the course of a second-cycle *in vitro* condition during anther culture. This was also evident as the AC lines showed the same banding pattern as their parental T_0 plants (data not shown). As expected, segregation in the AC-derived plants was observed because the recessive gametes, hemizygous for the *chi11* locus, had an equal likelihood to regenerate into green plantlets. On the basis of morphological features, ~60% of the total number of green plants were doubled haploids by spontaneous chromosomal doubling, and were morphologically normal. As seen from the western blot, the T_0 and the AC lines exhibited variation in the expression level of chitinase, which is in agreement with our earlier finding (Datta *et al.*, 1999b). The variability in the amount of 30-kDa protein, a proteolytic degradation product (Datta *et al.*, 2000) was also observed. The variation in the level of 28-kDa could be due to interaction of endogenous chitnase gene and the transgene under develop-

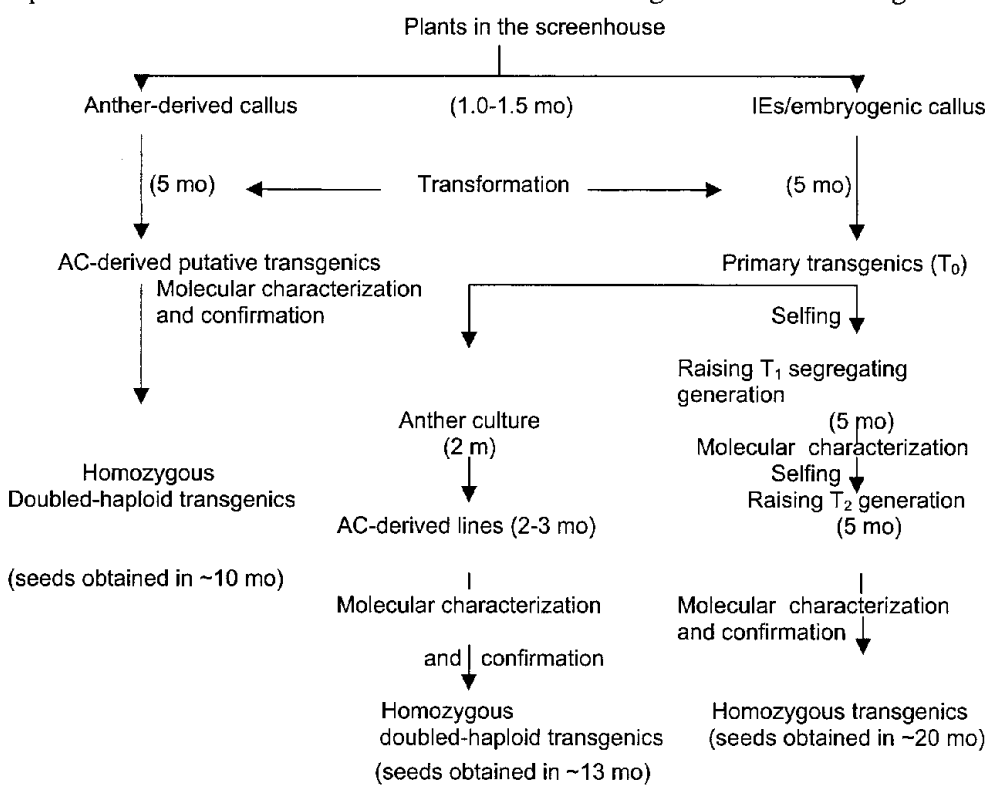

Fig. 10 A scheme showing the comparative time advantage in obtaining homozygous transgenics with and without the use of anther culture

mental and environmental control. AC-derived dihaploid lines, in general, had a higher level of overexpression of chitinase in terms of the activity compared with the control and the haploid transgenics (**Fig. 8**). This supports the earlier finding that hemizygous transgenics had chitinase levels one-half to one-fourth of the levels in homozygous transgenics. However, the line SHC1AC2, a dihaploid in our experiment, had lower chitinase activity than the control, which also substantiates the earlier reports in rice (Chereopornwattana *et al.,* 1999), sorghum (Zhu *et al.,* 1998), and tobacco (Hart *et al.,* 1992). This might possibly be due to the homology-dependent silencing, transgene inactivation, or co-supression of both endogenous and transgene chitinase (Jorgensen *et al.,* 1996; Meyer and Saedler, 1996). Moreover, the report on inactivation of the class I chitinase in homozygous transgenic tobacco suggested the transgene expression under environmental and developmental regulation (Meyer *et al.,* 1993; Meyer and Heidmann, 1994). The chitinase activity was the total of endogenous and transgene product. The activity of certain AC lines (e.g., SHC4AC2) was higher because of high level of endogenous chitinase in spite of low level of 35-kDa protein.

The efficiency of overproduction of chitinase was evident from the reaction of the doubled-haploid transgenics when challenged against sheath blight fungus. We found a strong positive correlation between the level of chitinase enzyme and the resistance in the transgenics (data not shown) as observed earlier (Lin *et al.,* 1995; Datta *et al.,* 2000). The introduction of the chitinase gene into different plants under the control of the CaMV 35S constitutive promoter has also been found to increase resistance to sheath blight fungus in greenhouse studies (Broglie *et al.,* 1991; Lin *et al.,* 1995; Datta *et al.,* 2000), as well as in the field (Grison *et al.,* 1996). Our main objective was to develop the homozygous transgenics in shortest possible time. A general scheme depicting the comparative time advantage of transgenic research coupled with anther culture, and normal generation advancement of transgenics, is shown in **Fig. 10**. We obtained homozygous transgenics in about a year from the start of transformation till the confirmation of the AC-derived lines versus a minimum of 20-24 months required in the usual course of generation advancement. This would be of immense importance for meeting the immediate requirement of the rice growers of South and Southeast Asia where Swarna is an important cultivar. Moreover, under optimized conditions, homozygous transgenic lines could be made available before breakdown in resistance occurs because of the evolution of a new pathotype or biotype. In addition, the homozygous transgenics, apart from their possible direct release as varieties in areas of endemic sheath blight infestation, could also be used successfully as resistant donor parents in a cross breeding program where no resistant sources have yet been available among cultivated rice or its wild relatives.

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