Prospects of Engineering Virus Resistance in Rice: Achievements and Opportunities

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Abstract

Rice tungro disease is a major biotic stress affecting rice in South and Southeast Asia. Two viruses, Rice tungro bacilliform virus (RTBV), a DNA virus and Rice tungro spherical virus (RTSV), an RNA virus are found to be associated in tungro infected leaves. In the absence of well-defined genetic sources of resistance against RTBV and RTSV, a transgenic approach, based on RNA-interference (RNAi), using RTBV and RTSV genes, is attempted to obtain resistance in rice. RNAi is an inherent defense mechanism in plants, which is activated by the presence of RNA having strong secondary structures, such as hairpin or as double-stranded, and which brings about sequence-specific degradation of homologous mRNA, resulting in silencing of the gene expression. Viruses are targets of RNAi response in plants, which can generally overcome the defense response by viral suppressor proteins, resulting in disease. Constitutive expression of viral RNAi-inducing DNA constructs strengthens the anti-viral RNAi response and results in viral resistance, as demonstrated in many cases. The same principle was applied to develop resistance against RTBV and RTSV in rice. DNA fragments representing approximately 300 bp of RTBV DNA and RTSV cDNA were used to make constructs capable of giving rise to hairpin viral RNA (representing fused RTBV and RTSV genes) under strong constitutive promoters in a binary vector. These vectors contained the hygromycin phosphor-transferase gene, capable of imparting resistance to plant tissue against 50 mg/L hygromycin, which can act as the selection marker during transformation of rice tissue; the marker genes having flanking loxP sites, capable of being excised to remove the gene upon crossing with a rice line carrying the cre gene. This incorporates the well-known Cre-Lox system of marker gene removal for obtaining marker-free rice lines. Transgenic rice lines were generated using two different combinations of RTBV and RTSV genes carried in the above constructs, utilizing Agrobacterium-mediated transformation. Transgenic plants, selected for resistance to hygromycin were obtained and grown till maturity. The seeds were sown to obtain T_1 lines were analyzed for the expression of the viral genes. Resistance to RTBV and RTSV is under evaluation.

Keywords: Tungro, virus, resistance, RNAi, transgenic rice

Introduction

Rice tungro disease (RTD) is a viral disease of rice, prevalent in south and southeast Asia. RTD has been estimated to cause an annual loss in excess of 10^9 US dollars in the affected countries (Herdt 1991) and about 2% loss of production in India, although, at the regional level, the losses could be significant (Muralidharan et al 2003). RTD was first reported in India in the late sixties (Raychaudhury et al 1967a; Raychaudhury et al 1967b), soon after the first description of the disease from the International Rice Research Institute, Philippines (Rivera and Ou 1965). Since then, the disease has been intensively studied in India and elsewhere, resulting in the accumulation of significant knowledge regarding the causative viruses, transmission, alternate hosts, pathological and biochemical changes and resistance genes (Azzam and Chancellor 2002). In the last decade several attempts have also been reported to engineer resistance against the disease and to further characterize resistance genes. We summarize all these contributions and new developments aimed at obtaining RTD resistance and list a few approaches, which may be adopted in the future to manage and control this disease.

Review of recent advances in research

General aspects

Although RTD is known by various names in different countries of Asia, its characteristic symptoms remain the same, namely yellow-orange foliar discolouration and stunting. RTD is spread by an insect vector found in abundance in rice fields, the green leafhopper (GLH), *Nephotettix virescens* (Ling 1974). Two viruses are

found associated with RTD, the bullet shaped *Rice tungro bacilliform virus* (RTBV) and the isometric *Rice tungro spherical virus* (RTSV), having DNA and RNA as the genetic materials respectively (Jones et al 1991). Studies have clearly revealed that GLH can transmit RTSV independent of RTBV, but the latter is always transmitted with RTSV (Cabauatan and Hibino 1985). RTSV, on its own, causes mild stunting, whereas RTBV causes the typical yellow-orange foliar symptoms in affected plants. In the presence of both the viral components, the symptoms are accentuated. Hence, it was concluded quite early on, that RTSV provides the transmission functions for the viral complex, but the symptoms are caused mainly by RTBV (Hibino and Cabauatan 1987; Cabauatan and Hibino 1988). Experimental evidence for this hypothesis became available when it was shown that a cloned RTBV genome was capable of infecting rice plants, independent of GLH, if introduced through *Agrobacterium* as a cloned DNA in a binary plasmid (Dasgupta et al 1991). The precise mechanism by which RTSV provides the transmission functions are yet unknown.

Gene functions and variability of RTBV

RTBV belongs to the virus family *Caulimoviridae*, genus *Tungrovirus* and is its sole member. A common feature of all the members of the family is a circular, double-stranded DNA genome of approximately 7- 8 kbp, with genes arranged only in one direction. A conserved short sequence representing the binding site for a tRNA primer is present in all the members of this family, which primes cDNA synthesis on a full-length RNA template; an essential step in the replication of the DNA genome. For this unusual step in the viral life cycle, all members of this family are also known as pararetro viruses. All RTBV genomes analyzed todate are about 8 kbps in size and have four open reading frames (ORFs); of which only ORFIII have been assigned functions, based on biochemical analysis of the expressed proteins. ORFIII encodes a large poly-protein, which has domains exhibiting functions of a movement protein, coat protein (CP), protease and reverse transcriptase-RNAse-H (Laco and Beachy 1994; Marmey et al 2005); an arrangement conserved in all pararetro viruses (Hay et al 1991). There is very little information on the functions of the proteins encoded by ORFs I, II and IV, other than ORF II, which interacts with RTBV CP, both in vitro as well as in vivo, the interacting domains being essential for infectivity of the virus, which point towards the possible essential nature of such interactions (Herzog et al 2000). Comparison of the DNA sequences of the full-length RTBV genomes determined almost a decade ago clearly showed that they can be grouped into two; the South Asian and the Southeast Asian groups; the isolates from India and Bangladesh falling in the former and those from the countries of Myanmar to Philippines, falling in the latter (Nath et al 2002). ORF IV, which is present only in the Genus *Tungrovirus*, shows the highest sequence heterogeneity between the two groups of RTBV, as compared to the rest of the ORFs. More recently, this division has been reinforced by the analysis of newer full-length (Sharma and Dasgupta 2012; Banerjee et al 2012) and partial RTBV sequences (Mangrauthia et al 2012a), which have become available from new regions in India.

Gene functions of RTSV

RTSV, a member of the family *Secoviridae*, has a single-stranded positive-sense RNA as its genome and encodes a large poly-protein, which can potentially give rise to three CPs and a replicase, in addition to several other proteins (Shen et al 1993). Although the presence of two additional small ORFs as the 3' end of the RNA was initially reported, it was not confirmed in subsequent studies (Verma and Dasgupta 2007). The roles of the rest of the proteins encoded by the RTSV genome are still obscure. In contrast to RTBV, RTSV sequences from across south and southeast Asia show high sequence conservation (Verma and Dasgupta 2007).

Role of the viruses in RTD

RTBV and RTSV were known to be associated with RTD from 1975-76, a causative role of these two viruses in RTD has been not been shown. Because of the lack of mechanical transmission and the absolute requirement of GLH and RTSV for transmission of RTBV, the demonstration of its role in RTD was attempted using "agroinoculation", a method in which the viral DNA is cloned in a binary vector and introduced to the plant via *Agrobacterium*. Rice plants not only became infected with RTBV after agroinoculation using full-length RTBV DNA and showed typical, but mild symptoms of RTD, the virus could also be transmitted further by GLH (Dasgupta et al 1991). The agroinoculated plants showed a recovery later. This suggested for a possible causative role of RTBV in tungro disease.

Conventional methods to attain RTD resistance

Early attempts to manage RTD focused on the availability of resistance genes in the rice germplasm and their transfer to popular cultivated ricevarieties using classical breeding methods. Extensive screening of the available rice germplasm revealed a moderately large number of rice varieties, which showed resistance or tolerance to RTSV, but very few against RTBV (reviewed in Azzam and Chancellor 2002). No information was available on the nature of these genes or their mode of action and only in very few cases was

their chromosomal locations mapped by the help of molecular markers. In addition, in the absence of effective methods to discriminate between the resistance to RTBV and RTSV, and resistance to GLH, the possibility of the latter could not be ruled out. GLH resistance is believed to be transient in nature and is generally based on the presence of an anti-feedant. Such resistance may be easily broken under heavy GLH pressure (Manwan et al 1985).

Non-conventional methods to attain tungro resistance

Targeting RTSV

The earliest report at engineering RTD resistance used the classical CP-mediated resistance strategy. The three CPs of RTSV, either singly or in various combinations, were expressed in rice under constitutive promoters. The plants contained integrated copies of the CP genes. Expression of the various CPs was detectable by the accumulation of the corresponding transcripts. When challenged with viruliferous GLH, some lines escaped infection and there was a significant delay in the buildup of RTSV (Sivamani et al 1999). Subsequently, the replicase (rep) gene of RTSV was used to engineer resistance by inserting it in sense as well as in anti-sense orientation. Upon challenge with viruliferous GLH, a significant proportion of the plants were resistant to RTSV, even against multiple isolates, in the lines containing the rep gene in anti-sense and in those containing in sense orientation. The investigators also reported a low transmission of RTBV from the transgenic lines, indicating that these transgenic plants would form an effective barrier to the spread of RTD (Huet et al 1999).

Targeting RTBV

Early attempts at achieving resistance against RTBV, by expressing various viral proteins, were unsuccessful; probably because of inappropriate expression levels of the transgenes (Azzam and Chancellor 2002). Subsequently, Ganesan et al (2009) reported CP-mediated resistance against RTBV. The transgenic rice lines accumulated the viral CP and displayed low levels of RTBV upon challenge inoculation with viruliferous GLH. The inoculated plants showed only mild symptoms of RTD. This was followed by attempts to obtain RTBV resistance based on RNA-interference (Ratcliffe et al 1999), an inherent defense mechanism in plants, the components of which are conserved in a wide variety of organisms. Tyagi et al (2008) demonstrated that transgenic rice designed to express a double-stranded RNA against RTBV ORF IV, upon challenge inoculation with viruliferous GLH, accumulated RTBV DNA much slowly, as compared to the non-transgenic control lines. These plants had very low levels of the transcripts for the transgene, but accumulated the corresponding small RNA. These properties are a hallmark of RNA-interference, which is triggered by double-stranded RNA. Similar to the previous report, these plants showed mild stunting and no yellowing, unlike the non-transgenic control plants inoculated in the same manner. The evidence indicated strongly that RNA-interference against RTBV could reduce its level of accumulation, which results in a significant amelioration of RTD symptoms. Subsequently, selected lines of transgenic plants were used to introgress the transgene into a number of popular rice varieties by back-cross breeding, using molecular markers. Testing of two such lines at the BC_2 stage has showed the transfer of the resistant trait to the progeny, while retaining the agronomical qualities of the parental lines (Roy et al 2012). This represents the first targeted engineering of RTBV resistance in rice, which needs to be tested under field conditions in the areas endemic to RTD and also against the south east Asian group of RTBV.

A different approach to achieve RTBV resistance was reported by Dai et al (2008). The group had earlier reported that rice transcription factors, RF2a and RF2b, which are required for the normal growth and maturation of rice plants, bind strongly to the RTBV promoter (Dai et al 2004). The hypothesis put forward them was that, upon RTBV infection, these two factors are sequestered by the RTBV promoter that resulted in a fall in its availability required for the normal expression of genes for the growth and development of the plant leading to the appearance of symptoms of tungro. Using the same logic, the authors over-expressed RF2a and RF2b in rice plants and these lines, apart from being phenotypically normal, when challenged with viruliferous GLH, remained largely symptom-free. This novel approach, although shown to work under laboratory conditions, needs to be further tested, especially using the south Asian group of RTBV.

Characterizing resistance genes

RTSV resistance genes are fairly widespread in the rice germplasm. Taking this knowledge further, the genetic nature of RTD resistance was dissected in the cultivar Utri Merah (Encabo et al 2009). By using near isogenic lines derived from this line and the susceptible cultivar TN-1, the RTD resistance was linked to the suppression of interacting RTBV and RTSV, but the suppression trait was inherited separately. Using a map-based cloning approach, RTSV resistance gene in UtriMerah was located on a DNA fragment, which was cloned and sequenced. Analysis of the corresponding fragments from a number of resistant and susceptible rice varieties suggested that the RTSV resistance gene allele (*tsv1*) corresponded to a eukaryotic translation initiation factor (eIF4G), which, upon being mutated prevented the

accumulation of RTSV (Lee et al 2010). The above has been an exciting development and it can be hoped that this information and the associated genetic markers would be widely used in the future to introgress this allele into cultivated rice varieties.

New developments towards management

Genome analysis of new isolates of RTBV and RTSV

The single promoter region of RTBV, driving the expression of the four ORFs, has been the subject of intense investigation in the past, using the isolates from Philippines (Dai et al 2006). An Indian RTBV isolate was shown by Mathur and Dasgupta (2007) to have a very different architecture as far as the functional domains of the promoters were concerned. Not surprisingly, they reported novel expression patterns of the Indian RTBV promoters and their derivatives, especially negative expression elements in the downstream regions. The work has been expanded further to show the orientation and position independence of the negative element and its capacity to silence heterologous promoters (Purkayastha et al 2010). The importance of these expression elements on the pathology of RTD is an area waiting to be explored. A recent report on an isolate from Tamil Nadu southern India, an area which has seen emergence of RTD in a big way in the last few years, shows that the RTBV genome is likely to have undergone recombination (Sharma and Dasgupta 2012), illustrating the general principles of natural population dynamics of viral genomes. Although the isolate shows no change in its basic properties, one needs to keep a watch on the emergence of isolates, which might be products of recombination resulting in altered pathogenicity. Banerjee et al (2011a) by analyzing the sequence of a new RTBV isolate from West Bengal (Chinsurah) has concluded that the evolution of RTBV strains is strongly dependent upon the geographical region. This conclusion was reached by looking for conserved motifs of the large intergenic region (Banerjee et al 2011b). Mangrauthia et al (2012a) compared the sequences of ORF I, ORF II and ORF IV from two new isolates of RTBV; Cuttack and Puducherry with those already available in the database and concluded that the sequence elements and motifs are conserved to a high degree. Another recent study from the same group has compared the CP sequences of two new RTSV isolates (Cuttack and Puducherry) with those already available in the database and concluded that, although at the nucleotide levels compared, the Indian RTSV isolates could be thought to fall into two groups, the differences in the protein level were insignificant, thereby strengthening the earlier view of tight conservation of RTSV sequences (Mangrauthia et al 2012b).

Improved design of RNAi vectors

RTBV and RTSV depend upon each other as causative agents for RTD, and hence it is generally believed that a resistance strategy against one would be sufficient to control both. However, a joint resistance approach against both the viruses is expected to result in enhanced assurance against RTD, to preempt the emergence of new viral strains. With a view to combine RNAi against RTBV and RTSV, hairpin loop constructs have been designed, combining fragments derived from both RTBV and RTSV. In addition, to facilitate removal of the antibiotic selection marker, the well-known *Cre-lox* system has been incorporated (Sharma et al, unpublished). In this construct, the antibiotic selection marker, *hygromycin-phosphotransferase* (*hpt*) is flanked by two *lox* sites, which will result in the excision of *hpt* upon sexual crossing with a *cre*-expressing line (Fig. 1).

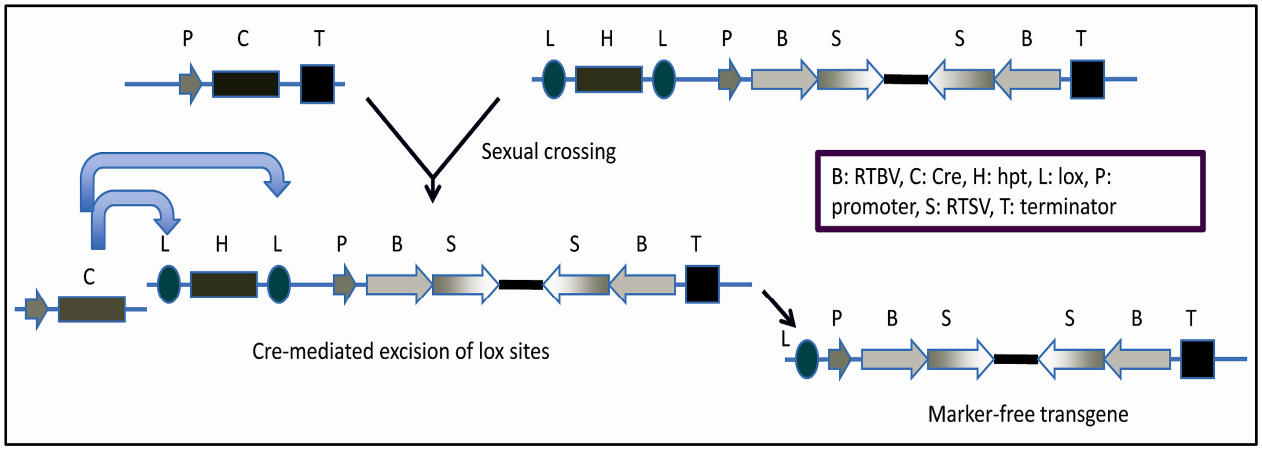

Figure 1. Schematic diagram illustrating the construct for achieving resistance against rice tungro virus disease by using RNAi against both RTBV and RTSV and the process of marker removal following crossing with a Cre-expressing plant.

This is expected to improve the consumer acceptability of the rice lines. Keeping this strategy in mind, two sets of gene combinations- RTBV RT-RNAseH, and RTSV Rep and RTBV promoter-RTSV Rep, have been obtained in the *lox*-containing binary vector. The hairpin constructs are placed downstream to the constitutive CaMV 35S promoter. Currently, transgenic rice plants have been obtained by *Agrobacterium*-mediated transformation and are in the T_2 generation. The plants have been checked for their transgenic status by PCR. In parallel, transgenic plants expressing the *cre* gene have also been obtained. The testing of the resistance of the plants to RTD is still awaited.

New opportunities to develop RTD resistance

An exciting new approach towards developing RTD resistance could target the process of transmission of the viral complex by GLH. Early evidences pointed towards a non-viral factor associated with RTSV (Cabauatan and Hibino 1985). The unraveling of the hypothetical helper factor of RTBV transmission will depend upon the development of membrane feeding methods for GLH, where the acquisition of the viral particles could be analyzed at the biochemical level. Other approaches should include the characterization of new resistance genes against both RTBV and RTSV. Using the vast and varied rice germplasm existing in India, such an exercise should yield useful genes, if planned and executed in a time-bound, interdisciplinary and mission-mode manner, using tightly linked molecular markers. Lastly, the inter-relationship between RTBV and RTSV should be analyzed in detail; how they interact within the rice plant and within the GLH vector, to cause this unique and important disease.

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