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**A single copy of a virus-derived transgene encoding hairpin RNA  
gives immunity to barley yellow dwarf virus.**

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## **Abstract**

The PAV serotype of barley yellow dwarf virus (BYDV-PAV) is the most damaging virus of cereals both in Australia and worldwide. Natural resistance genes against the virus give inadequate control and previous attempts to introduce synthetic resistance genes have been disappointing. The transformation of cereals with BYDV coat protein or polymerase genes has produced a few virus-resistant plants but the resistance has been weak, unstable or has failed to segregate as expected.

In an attempt to generate barley with protection against BYDV, Golden Promise barley was transformed with a transgene that was designed to produce hairpin (hp)RNA from BYDV-PAV sequences. The majority of the resulting lines contained a single copy transgene insert and were rated as immune because the virus could not be detected in the challenged plants by ELISA nor recovered by aphid feeding experiments.

In the field, BYDV-PAV is sometimes associated with the related luteovirus Cereal yellow dwarf virus (CYDV-RPV). When the transgenic plants were challenged with BYDV-PAV and CYDV together, the plants were susceptible to CYDV but immune to BYDV-PAV. This shows that the immunity is sequence specific and not broken down by the presence of CYDV. It also suggests that the immunity will be robust in the field and very useful in minimizing losses in barley production due to BYDV-PAV.

## **Introduction**

BYD disease comprises two subgroups of viruses - BYDV (Barley yellow dwarf virus) and CYDV (Cereal yellow dwarf virus), and is the major viral disease of barley in Australia, reducing yield by an average of 15% per annum (Lister and Ranieri, 1995). BYDD can also affect plant height, grain size and grain quality, such that grain is suitable only for animal feed rather than malting.

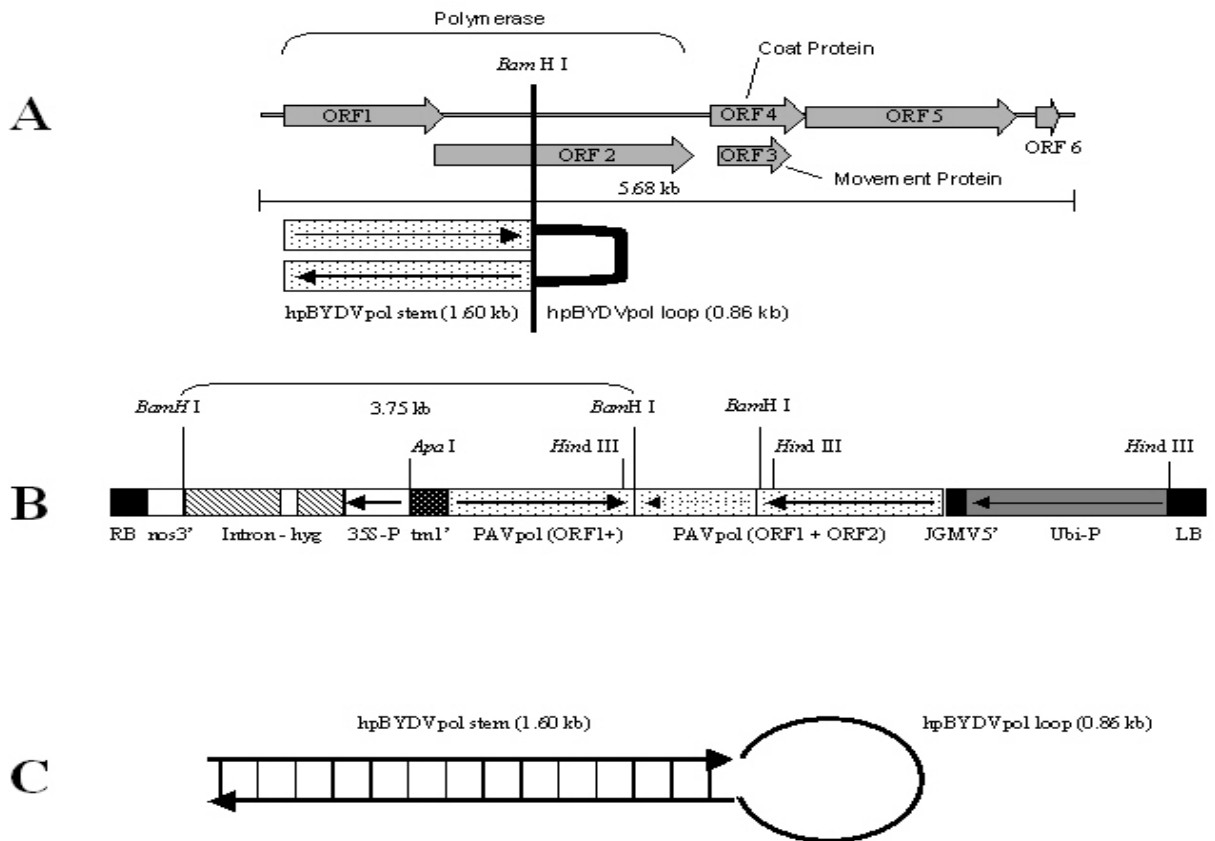
Sources of natural resistance to BYDV and CYDV are rare (for reviews see Barker and Waterhouse 1999; Burnett *et al.*, 1995). In barley, the *Yd2* gene (Paltridge *et al.*, 1998), originally identified in Ethiopian concessions (Schaller *et al.*, 1964), can confer resistance against BYDV-PAV, but its effectiveness varies depending on the genetic background of the plant and growth conditions (Larkin *et al.*, 1991). BYDV still replicates in plants containing the *Yd2* gene.

Recently, we discovered that virus immunity and posttranscriptional gene silencing (PTGS) can be induced in plants using transgenes that encode double stranded (ds) or self-complementary "hairpin" (hp) RNA (Waterhouse *et al.*, 1998; Wang & Waterhouse, 2000; Smith *et al.*, 2000). This protection/silencing appears to operate through sequence-specific RNA degradation similar to that of RNA interference (RNAi) in *Drosophila* (Zamore *et al.*, 2000). Here we describe the transformation of barley plants with a construct that encodes a hpRNA containing the polymerase gene sequences from the major serotype of BYDV, PAV. Our results indicate that this transgene confers immunity to BYDV-PAV on the plants and, where it occurs as a single locus, it is inherited in a simple Mendelian manner.

## Materials and Methods

A gene construct (hpBYDVpol) was made in which a hairpin RNA, containing BYDV-PAV polymerase gene sequences, is transcribed under the control of the maize ubiquitin promoter (Fig. 1). Further details for the construction of hpBYDVpol and the transformation of *Hordeum vulgare* L. cv. 'Golden Promise' with this gene are provided by Wang *et al.* (2000). Aphids (*Rhopalosiphum padi*) were used to infect barley plants with an Australian isolate of BYDV-PAV, an Australian isolate of CYDV-RPV and an Australian B/CYDV-MIX isolate which contained both BYDV-PAV and CYDV-RPV (Waterhouse *et al.*, 1986). Virion accumulation was measured by enzyme-linked immunosorbent assay (ELISA) (Xin *et al.*, 1988).

**Figure 1. (A) Genome map of BYDV-PAV showing regions used to generate hpBYDVpol. (B) Design of hpBYDVpol construct. (C) Diagram of self-complementary (hairpin) RNA produced by hpBYDVpol.** RB: right border; nos 3': nopaline synthase 3' region; 35S-P: cauliflower mosaic virus 35S promoter; tml1': tumour morphology large gene 3' region; PAVpol: BYDV-PAV polymerase gene sequence; JGMV5': Johnson grass mosaic virus 5'untranslated region; Ubi-P: maize polyubiquitin gene promoter; LB: left border.



Southern analysis (Lagudah *et al.*, 1991) used a radio-labeled probe from a 1.1 kb *hpt* (hygromycin resistance gene) fragment. PCR analysis was performed using oligonucleotide primers amplifying a 626 nt fragment from the spacer loop between sense and antisense sequences of the transgene.

# Results and Discussion

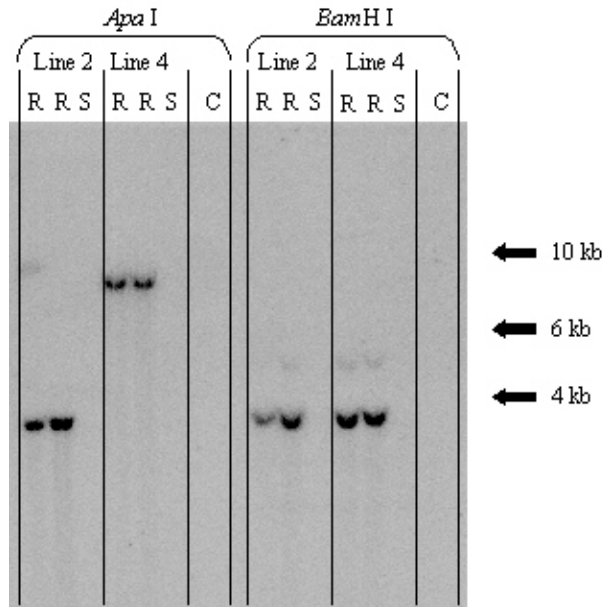
## Transformation and Analysis of T<sub>0</sub> plants

Using this construct and an *Agrobacterium*-mediated transformation system, an overall transformation efficiency of 13% was achieved resulting in 38 independent transgenic barley plants. Southern analysis indicated that 19 plants carried a single transgene copy, 12 contained two copies and 7 had three or more copies (data not shown). When 25 of the T<sub>0</sub> plants were inoculated with BYDV-PAV, 9 of them appeared highly resistant as they supported little or no virus replication when measured by ELISA (data not shown). Two single copy lines (2 and 4) were selected to investigate the inheritance of the hpBYDVpol transgene and of BYDV-PAV resistance.

## Transgene inheritance and virus immunity in hpBYDVpol Lines 2 and 4

Fourteen T<sub>1</sub> plants of Lines 2 and 4 were challenged with BYDV-PAV and monitored for virus symptoms and virion accumulation 21, 28, and 42 days after inoculation. The progeny of lines 2 and 4 conformed to a segregation ratio of 3 : 1 (highly resistant : susceptible), suggesting the presence of a single dominant transgene locus in each line, and Southern analysis (Fig. 2) revealed that the loci each appear to contain a single transgene. The inheritance of the hpBYDVpol transgene in these plants was examined by PCR amplification of a 626 bp fragment of DNA from the transgene. In both lines (Fig. 3), the inheritance of hpBYDVpol correlated perfectly with lack of virus symptoms and resistance to BYDV infection. Plants that were symptomless and contained undetectable virus levels 21 days after inoculation, remained this way throughout the six weeks of analysis (Fig. 3A), whereas those plants without the hpBYDVpol transgene showed virus symptoms and virion accumulation at all three time-points. The co-segregation of virus accumulation with the absence of the transgene was also evident in the grain yield from the individual progeny plants. In Line 2, the average grain yield from the nine hpBYDVpol-containing T<sub>1</sub> progeny was 29.7±1.8 g compared to a yield of 20.9±1.5 g from the six transgene-free progeny. Twenty-eight days after inoculation, the ELISA level in plant 12 of Line 4 (Fig. 3) suggested that some virus accumulation had occurred. However, the plant had undetectable levels of BYDV-PAV both one week before and two weeks after this time-point, and never developed virus symptoms. This suggests that there might have been a low level of virus replication, peaking at 28 days, which was then overcome by the transgene-induced resistance or that there was some contamination of the sample during analysis.

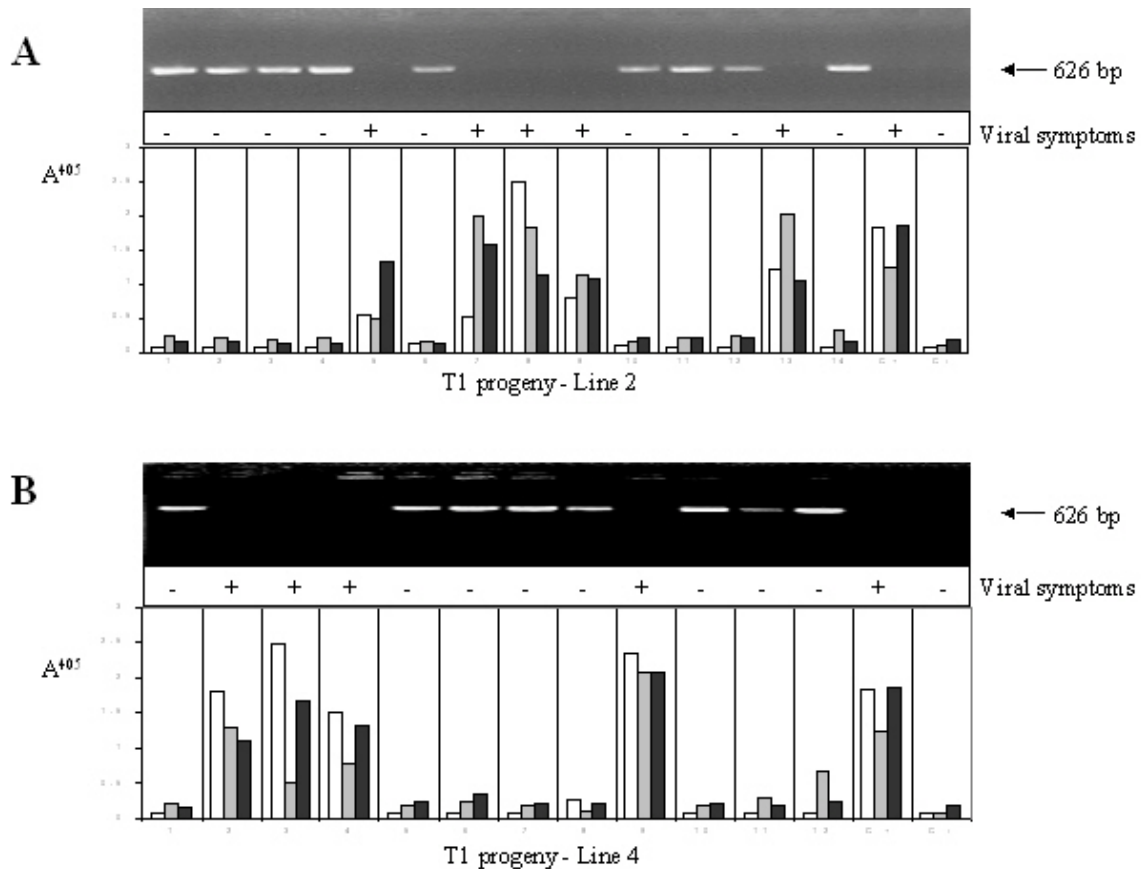
**Figure 2. Southern blot analysis of T<sub>1</sub> progeny of hpBYDVpol Lines 2 and 4.** DNA from two resistant (R) and one susceptible (S) T<sub>1</sub> plants for Lines 2 and 4, and for non-transgenic barley (C), was digested with *Apa* I or *Bam*H I, separated by electrophoresis, blotted to Hybond N+TM membrane and hybridized with a radioactively-labelled *hpt* probe. The number of bands in the *Apa* I lanes and the intensity of the 3.75 kb bands for the *Bam*H I lanes should indicate copy number.



## Specificity and robustness of the virus resistance

Although BYDV-PAV is the major virus pathogen of cereals in the field, CYDV-RPV can also be present. Indeed the two viruses can occur as a complex and appear to have a synergistic effect on symptom severity. The viruses share common hosts, a common insect vector and many aspects of their biology. However, they are at opposite ends of the luteovirus spectrum in terms of sequence homology and genome organisation. To test whether the hpBYDVpol transgene also conferred protection against CYDV-RPV, approximately 15 T<sub>1</sub> progeny from each of hpBYDVpol Lines 2 and 4 were challenged with BYDV-PAV and another 15 of each line with CYDV-RPV. Measuring the virus accumulation in these plants by ELISA revealed that, as before, both lines segregated 3:1 for BYDV-PAV resistance. However, all of the plants inoculated with CYDV-RPV were fully susceptible to the virus. These results indicate that the resistance conferred by the hpBYDVpol transgene is specific to BYDV-PAV. This might have been expected as there is only 34% homology between nucleotide sequence in hpBYDVpol and the corresponding region in CYDV-RPV.

**Figure 3. Relationship between virion accumulation and inheritance of the hpBYDVpol transgene.** Virus levels detected by ELISA 21 (white bar), 28 (grey bar) and 42 (black bar) days after inoculation in (A) 14 T<sub>1</sub> progeny of Line 2 and (B) 12 T<sub>1</sub> progeny of Line 4. Displayed above each histogram is a symbol representing the severity of viral symptoms in mature plants and an agarose gel containing PCR products from the corresponding plant samples; the presence of a 626bp product indicates the amplification and detection of the hpBYDVpol transgene.



It has recently been shown that some viruses have the capacity to inactivate PTGS (Voinnet *et al.* 1999). Therefore, it was important to determine whether infection by CYDV-RPV could inactivate the hpBYDVpol-mediated BYDV-PAV resistance. To test this, 15 T<sub>1</sub> progeny from hpBYDVpol Line 2 were inoculated with the B/CYDV-MIX isolate, a virus complex containing BYDV-PAV and CYDV-RPV. The plants were subsequently tested using species-specific ELISA for accumulation of BYDV-PAV and CYDV-RPV and by PCR for inheritance of the transgene. The results (Fig. 6) showed that CYDV-RPV replicated to high levels in all 15 plants but that the 12 plants inheriting the hpBYDVpol transgene were resistant to BYDV-PAV (data not shown). This indicates that the resistance to PAV is not compromised by replication of CYDV-RPV and further confirms the 3:1 (BYDV-PAV resistance: susceptible) segregation ratio.

## Recovery of virus from virus-challenged hpBYDVpol plants

Although BYDV-PAV-challenged hpBYDVpol-plants contain extremely low levels of BYDV-PAV antigen, they might contain sufficient virus to be acquired by aphids and thus be of ecological significance. To examine this, we attempted to recover infectious virus from T1 progeny plants from lines 2 and 4 that had been previously challenged with either BYDV-PAV or B/CYDV-MIX. Virus-free aphids were fed (for three days) on the plants ten weeks after the initial challenge and then transferred to healthy test plants. Whereas the test plants became infected with BYDV-PAV from aphids fed on BYDV-PAV or B/CYDV-MIX challenged wildtype or non-transgene segregant plants, none of them was infected with BYDV-PAV from aphids fed on similarly challenged plants containing the hpBYDV-PAVpol transgene. However, aphids did recover CYDV-RPV from hpBYDVpol plants challenged with the B/CYDV-MIX mixture. Taken altogether, the data show that BYDV-PAV-challenged hpBYDVpol plants contain no biologically active virus and should be regarded as immune to BYDV-PAV (Fig. 4).

**Figure 4. Reaction of transgenic and non-transgenic barley to BYDV.** Two T1 hpBYDVpol immune plants of Line 2 (left) and two nontransformed plants (right).



## Conclusion

We have generated barley plants containing transgenes encoding a hpRNA derived from BYDV-PAV polymerase sequences. Over one-third of these independently transformed plants have extreme resistance to BYDV-PAV. Furthermore, some of the plants have a single transgene that is inherited, along with virus immunity, in a simple Mendelian manner. This is a significant advance over previous attempts to produce transgenic cereals with protection against BYDV. While some attempts (McGrath *et al.*, 1997; Koev *et al.*, 1998; Wang *et al.*, 2001) have produced oat or barley plants with resistance (reduced virus replication) or tolerance (reduced virus symptoms but unimpeded virus replication) to BYDV, the inheritance of the resistance/tolerance has been variable. This has been further complicated by the complex transgene insertion patterns in such plants, especially those obtained using biolistic transformation.

Our BYDV-immune barley plants may have great potential for deployment in the field. However, a feature that must be considered before contemplating their widespread use is their interaction with other viruses. Some viruses have the capacity to enhance the replication and/or spread of co-infecting viruses and to inactivate PTGS (for example Vance 1991; Voinnet *et al*, 1999). Therefore, it was possible that infection by CYDV-RPV, which can co-infect with BYDV-PAV in the field, could enhance the replication and spread of BYDV-PAV or inactivate the hpBYDVpol-mediated BYDV-PAV immunity, thus disarming the plant's newly conferred protection. However, in our experiments, the protection against BYDV-PAV was not compromised by co-inoculation of the plants with CYDV-RPV and BYDV-PAV.

While it is reassuring that our plants should maintain their protection against BYDV-PAV in the field, it is our future goal to provide cereals with simultaneous protection against both BYDV and CYDV using hpRNA technology. Consequently, we have made several new hairpin constructs that we are presently using to transform barley. These constructs possess a number of features to increase their effectiveness and environmental safety. For example, they incorporate small segments of sequence from both BYDV-PAV and CYDV-RPV, selected on the basis of homology to provide protection across a range of isolates. In the unlikely event that one of these segments were to recombine with another invading virus they would provide no advantage because the segments do not encode a whole protein, or even a protein domain. In fact, not only should our new genes for transformation be highly effective against both BYDV and CYDV, they will not encode any additional protein in the plant. The new constructs also incorporate a recently developed technique to remove the selectable marker gene (usually either antibiotic or herbicide resistance) from transformed plants.

## Acknowledgements

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