Construction of plants resistant to TYLCV by using artificial zinc-finger proteins

Yoshihiro Koshino-Kimura, Kosuke Takenaka, Fumiya Domoto, Masayoshi Ohashi, Toshihide Miyazaki, Yasuhiro Aoyama and Takashi Sera

Department of Synthetic Chemistry and Biological Chemistry, Graduate School of Engineering, Kyoto University, Katsura, Nishikyo-ku, Kyoto 615-8510, Japan

ABSTRACT

Previously, we have demonstrated that plant DNA virus replication could be inhibited in *Arabidopsis thaliana* by using an artificial zinc-finger protein (AZP) and created AZP-based transgenic *A. thaliana* resistant to DNA virus infection. Here we apply the AZP technology to tomato yellow leaf curl virus (TYLCV) causing serious damage to an important agricultural crop, tomato. An AZP was designed to block binding of the TYLCV replication protein (Rep) to the replication origin. The designed AZP had much higher affinities towards the replication origin than did the Rep, and efficiently blocked Rep binding in vitro. The AZP gene was then introduced into a plant genome with the help of *Agrobacterium tumefaciens* to generate the transgenic plants. The current status of the construction of the AZP-expressing transgenic plants will be reported.

INTRODUCTION

Previously, we developed a novel approach to prevention of DNA virus infection by using artificial zinc-finger proteins (AZPs). AZPs that were designed by using our nondegenerate recognition code table to inhibit binding of viral replication proteins (Reps) to their replication origins prevented DNA virus infection in both plants and mammalian cells. One of the advantages of our strategy is the low or nonexistent risk of the emergence of resistant viruses. Because we target Rep binding to a viral replication origin, viruses need to mutate a DNA base(s) in a region recognized by the AZP and additionally to mutate Rep so that the Rep mutant can bind to the mutated replication origin. The probability of such synchronized double mutation should be extremely low.

For plant DNA viruses, we first demonstrated the concept by using beet severe curly top virus (BSCTV), which is known to severely infect *Arabidopsis thaliana*, as a model. We generated a six-finger AZP to block BSCTV Rep binding to its replication origin, and demonstrated that transgenic *Arabidopsis* plants expressing the AZP were completely resistant to BSCTV infection.

Here we apply the AZP technology to tomato yellow leaf curl virus (TYLCV). The virus spreads all over the world, and causes severe infectious disease in tomato, an important agricultural crop, and great damage to the production. Any effective methodology to prevent the virus infection has not been developed yet.

RESULTS AND DISCUSSION

The TYLCV Rep protein is known to bind to double strand DNA of the direct repeats (i.e., 5'-ATCGGTGTAATCGGTGTA-3') in the viral replication origin. To block the Rep binding to the direct repeats, a six-finger AZP was designed by using our nondegenerate recognition code table to bind to a 19-bp DNA (5'-GCAATCGGTGTATCGGTGTTACGTT-3') containing the direct repeats (underlined). The DNA encoding the AZP was cloned into a pET-21a plasmid (Novagen), and the corresponding protein was expressed in *Escherichia coli* and purified by ion-exchange chromatography. The Rep protein was prepared in a same manner.

The dissociation constants of the AZP and Rep were determined by gel shift assays. Under our experimental condition, half-maximal binding of the AZP was observed at much lower protein concentration than that of the Rep, indicating that the AZP has much higher affinity for the direct repeats than that of Rep. Furthermore, to examine whether or not the 6-finger AZP can block the Rep protein of TYLCV from binding to the direct repeats, competitive binding assays were performed. In the experiment, the AZP was first mixed with the DNA probe containing the direct repeats, and the Rep protein then was added to the binding mixture to simulate TYLCV infection in the transgenic plants constitutively expressing the AZP. In this experiment, we found that AZP inhibited binding of 1000-fold excess Rep to the direct repeats. This property of the AZP was same to that of an AZP previously designed for the Rep of BSCTV, which demonstrated efficient inhibition of the BSCTV infection to *Arabidopsis thaliana*.

Next, we introduced the AZP-expression cassette for plants into a plant genome to construct the AZP-transgenic plants. First, the expression cassette was constructed by a standard method and cloned into a binary vector. By infection with the *Agrobacterium tumefaciens* transformed with the resulting binary vector, the AZP-expression cassette was integrated into plant genomes. After selection of calli expressing the AZP on agar plates containing antibiotics, calli with shoot were transferred to shoot
elongation medium. When a stem was developed, the stems were transferred to rooting medium. The rooted plants were then subjected to molecular analysis to confirm that these plants contained the AZP-expression cassette in their genomes. After moisture acclimation, the AZP-transgenic plants were grown on soil until seeds were harvested.

CONCLUSION

The six-finger AZP designed for TYLCV blocked the Rep protein of TYLCV from binding to its replication origin efficiently in vitro. Since we had previously demonstrated that an AZP that inhibited binding of Rep of BSCTV could inhibit the virus replication in *Arabidopsis thaliana*, our results strongly support that the AZP designed in the present study will inhibit TYLCV replication in tomato. The construction of the AZP-expressing transgenic plants is now under way.

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*Corresponding author. E-mail: sera@sbchem.kyoto-u.ac.jp*