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Heterologous expression of antifreeze protein gene *AnAFP* from *Ammopiptanthus nanus* enhances cold tolerance in *Escherichia coli* and tobacco

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ABSTRACT

Antifreeze proteins are a class of polypeptides produced by certain animals, plants, fungi and bacteria that permit their survival under the subzero environments. *Ammopiptanthus nanus* is the unique evergreen broadleaf bush endemic to the Mid-Asia deserts. It survives at the west edge of the Tarim Basin from the disappearance of the ancient Mediterranean in the Tertiary Period. Its distribution region is characterized by the arid climate and extreme temperatures, where the extreme temperatures range from −30 °C to 40 °C. In the present study, the antifreeze protein gene *AnAFP* of *A. nanus* was used to transform *Escherichia coli* and tobacco, after bioinformatics analysis for its possible function. The transformed *E. coli* strain expressed the heterologous *AnAFP* gene under the induction of isopropyl β-D-thiogalactopyranoside, and demonstrated significant enhancement of cold tolerance. The transformed tobacco lines expressed the heterologous *AnAFP* gene in response to cold stress, and showed a less change of relative electrical conductivity under cold stress, and a less wilting phenotype after 16 h of −3 °C cold stress and thawing for 1 h than the untransformed wild-type plants. All these results imply the potential value of the *AnAFP* gene to be used in genetic modification of commercially important crops for improvement of cold tolerance.

1. Introduction

Antifreeze proteins (AFP) were firstly found in polar fishes, as well as insects living in freeze-zone, that permit their survival under the subzero environments by noncolligative effects that depress the freezing point of their blood or hemolymph but do not affect the melting points (Bildanova et al., 2013; Davies and Hew, 1990; DeVries, 1986; DeVries and Lin, 1977; DeVries and Wohlschlag, 1969; DeVries et al., 1970; Duman and DeVries, 1976; Duman et al., 2004; Graham et al., 1997; Hew and Yang, 1992; Knight and Duman, 1986; Marshall et al., 2004; Mueller et al., 1991; Raymond and DeVries, 1977; Stefen and Brian, 2004). The encoding genes of the AFPs were used to transform maize, tobacco, potato, tomato, wheat and *Arabidopsis*. However, the transgenic lines did not demonstrate significant improvement of cold tolerance when compared to the wild-types (Georges et al., 1990; Hightower et al., 1991; Holmberg et al., 2001; Huang et al., 2002; Kenward et al., 1993, 1999; Khanna and Daggard, 2006; Wallis et al., 1997; Wang et al., 2008). The noncolligative manner of the heterologous animal AFPs, as well as their expression rate, localization and stability might not be suitable for the cellular environments of the transgenic plants.

In plant kingdom, AFPs were detected in more than 30 species, and purified from winter rye (*Secale cereale*), bittersweet nightshade (*Solanum dulcamara*), peach (*Prunus persica*), carrot (*Daucus carota*), perennial ryegrass (*Lolium perenne*), wheat (*Triticum aestivum*), and Antarctic hair grass (*Deschampsia antarctica*) (Bravo and Griffith, 2005; Duman, 1994; Duman and Olsen, 1993; Griffith et al., 1992, 1997; Hon et al., 1994; John et al., 2009; Kumble et al., 2008; Meyer et al., 1999; Sidebottom et al., 2000; Smallwood et al., 1999; Tremblay et al., 2005; Urrutia et al., 1992; Wisniewski et al., 1999; Worrall et al., 1998; Yu and Griffith, 1999; Zhang et al., 2007). Most of these plants are cold-acclimated overwintering species and have an ability to survive freezing. The expression of their *AFP* genes is up-regulated in response to cold and other abiotic stresses (Hincha et al., 1997; Smallwood et al., 1999; Yu and Griffith, 2001). Although the thermal hystereses (the difference between melting point and freezing point) of the plant AFPs (0.2–0.5 °C) are usually lower than that of fish (0.7–1.5 °C) and insects (3–6 °C) (Scotter et al., 2006; Yu and Griffith,2001), their inhibition to ice growth and recrystallization is 10 to 100 times stronger than that of fish and insect AFPs (Ewart et al., 1999; Kuiper et al.,

Abbreviations: ADH, alcohol dehydrogenase; AFP, antifreeze proteins; CTAB, hexadecyltrimethylammonium bromide; EC, electrical conductivity; GUS, β-glucuronidase; HSP, heat shock protein; IPTG, isopropyl β-D-thiogalactopyranoside; LB, Luria-Bertani; qRT-PCR, quantitative real-time PCR; REC, relative electrical conductivity; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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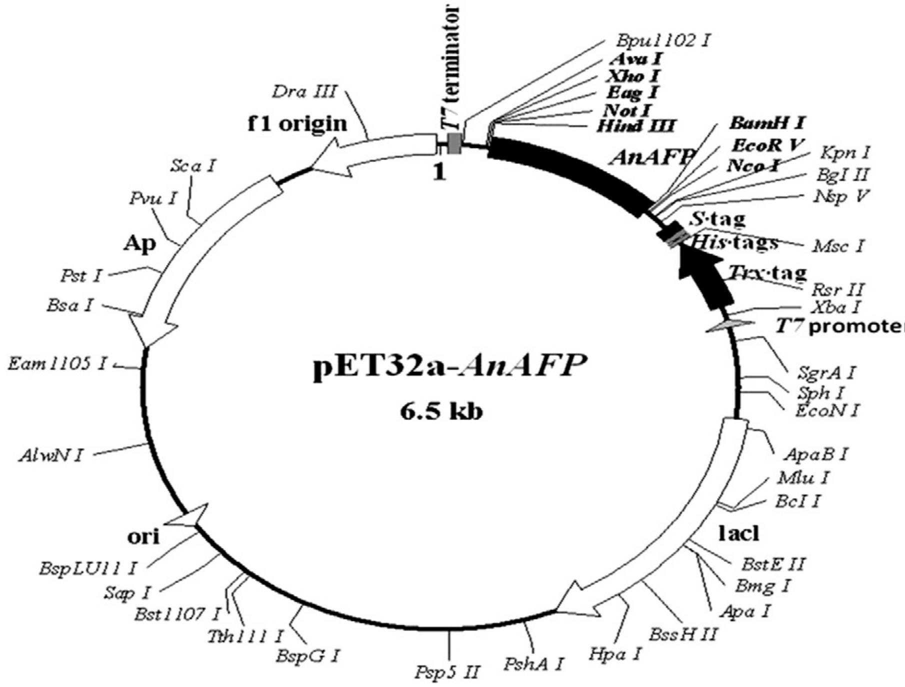

Fig. 1. Prokaryotic expression vector pET32-*AnAFP*. The *AnAFP* gene is under the control of the promoter and terminator of phage T7. The sequence immediately after the promoter encodes a leader peptide including a sulfoxide reductase protein tag (Trx-tag), two histidine selective tags (His-tag) and a S-tag for western blotting. The ampicillin resistance gene *Ap* is used as the selective marker.

2001; Pudney et al., 2003). The transgenic lines by plant *AFP* genes showed significant improvement of cold tolerance (Fan et al., 2002; Worrall et al., 1998; Xu et al., 2005; Zhang et al., 2010).

Ammopiptanthus nanus, one of the two relict species of the *Ammopiptanthus* genus (Leguminosae) (Cheng, 1959), is the unique evergreen broadleaf bush in the Mid-Asia deserts. It survives at the west edge of the Tarim Basin from the disappearance of the ancient Mediterranean in the Tertiary Period (Yan et al., 2000). Its distribution region is characterized by the arid climate and extreme temperatures, where annual precipitation is lower than 200 mm, annual evaporation is higher than 2000 mm, and dryness is usually more than 4. The extreme temperatures range from −30 °C to 40 °C. The habitat of *A. nanus* is usually stony and/or sandy slopes with poor soil quality and high salinity (Pan et al., 1992; Wang, 2005). To cope with these

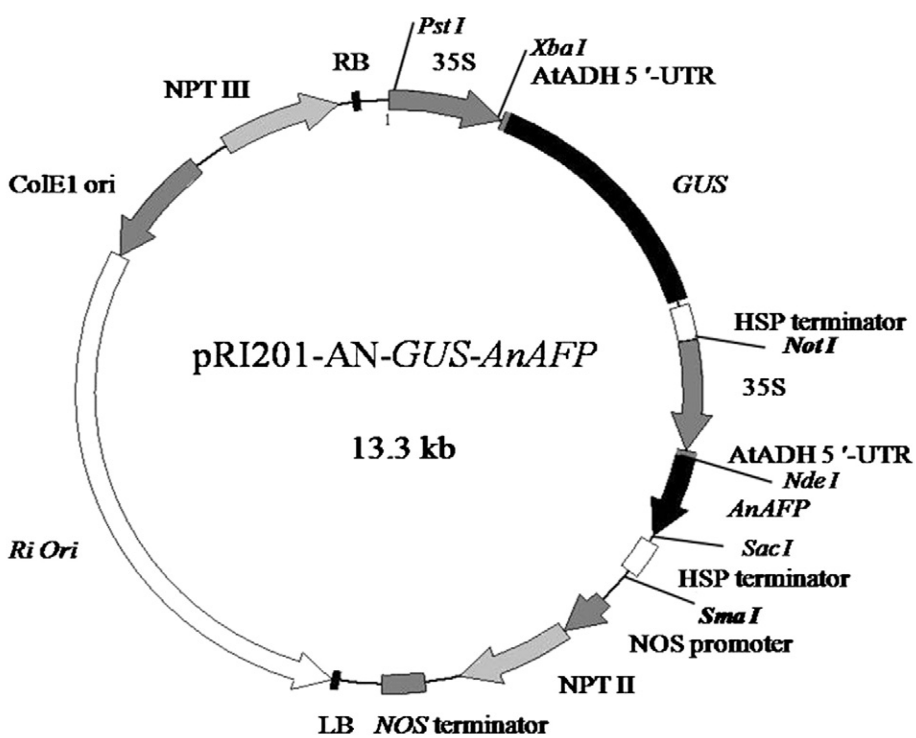

Fig. 2. Dicotyledonous plant expression vector pRI201-*GUS-AnAFP*. The *AnAFP* gene is under the control of the CaMV *35S* promoter, the *ADH* enhancer (5′-untranslated region of *Arabidopsis* alcohol dehydrogenase gene) and the *HSP* terminator, with the selective marker gene *GUS*.