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Male-sterile and cleistogamous phenotypes in tall fescue induced by chimeric repressors of *SUPERWOMAN1* and *OsMADS58*

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ABSTRACT

Since tall fescue (*Festuca arundinacea* Schreb.) is an anemophilous (wind-pollinated) grass species, male sterility is strongly desired for transgenic tall fescue to prevent pollen dispersal. To create male-sterile tall fescue, we applied Chimeric REpressor gene-Silencing Technology (CRES-T) based on rice *APETALA3* (*AP3*) and *AGAMOUS* (*AG*) orthologues that specify the formation of stamens. We fused the coding regions of rice *AP3* orthologue *SUPERWOMAN1* (*SPW1*), and rice *AG* orthologues, *Os12g0207000*, *Os01g0886200* and *OsMADS58*, respectively with the artificial sequence encoding the modified EAR-like motif repression domain (SRDX). We first introduced *Os12g0207000SRDX*, *Os01g0886200SRDX* and *OsMADS58SRDX* into rice for evaluation of their abilities to induce male sterility. The transgenic rice expressing *OsMADS58SRDX* had reiterated formation of lodicule-like organs instead of stamens and carpel, a typical phenotype of *ag* mutant. Thus, we found that *OsMADS58SRDX* was most suitable for our purpose. Next, we introduced *SPW1SRDX* and *OsMADS58SRDX* into tall fescue. Although the transgenic tall fescue did not have the stamen alterations seen in *SPW1SRDX* and *OsMADS58SRDX* rice, they either produced no pollen or produced immature pollen; thus, the anthers were not dehiscent and the plants were male-sterile. In addition to the male sterility, *SPW1SRDX* tall fescue showed a cleistogamous (closed) phenotype in which anthers were not observed outside the glumes, with thin, abnormally elongated lodicules. Some lines of *OsMADS58SRDX* tall fescue showed a cleistogamous phenotype in which the lodicules were homeotically transformed into lemma-like organs. In both cases, cleistogamous phenotype was associated with morphological changes to the lodicules. We also obtained a mild phenotype of *OsMADS58SRDX* tall fescue, which exhibited only the male sterility. In this study, we produced novel male-sterile phenotypes using chimeric repressors and thus suggest CRES-T as a tool for transgenic improvement of forage and turf grasses.

1. Introduction

One of the risks in the cultivation of transgenic plants is the possibility that transgenes will be spread by the plants crossing with sexually compatible weed species. Since most forage and turf grasses are cross-pollinated and anemophilous (wind-pollinated) species, transgenic grasses are likely to disperse transgenic pollen into the environment. Male sterility is a possible approach to prevent pollen flow. Cytoplasmic male sterility might be an effective strategy, but it is available only for limited species.

The "ABC model" has been provided to explain how genes control identity of four floral organs (sepals, petals, stamens and carpels) in the dicots *Arabidopsis thaliana* and *Antirrhinum majus* [1–3]. Most of the ABC genes encode MADS-box transcription factors. Since mutation of the ABC genes causes homeotic transformation of floral organs, modification of the ABC genes makes it possible to improve floral traits.

A combination of B- and C-class genes specifies the formation of stamens. *APETALA3* (*AP3*), a B-class gene of *Arabidopsis*, regulates stamen and petal identity [4]. In *ap3* mutant, the stamens and petals were homeotically transformed into carpels and sepals, respectively [4]. *AGAMOUS* (*AG*), a C-class gene of *Arabidopsis*, plays an important role in organ identity of stamens and carpels, the repression of A-class genes, and floral meristem determinacy in *Arabidopsis* [2,5–7]. In *ag* mutant, the stamens were homeotically transformed into petals, and central carpels were replaced by another *ag* flower [2,5]. Thus, the loss-of-function phenotype of each *AP3* and *AG* resulted in male-sterile flowers.

Although structure of flowers in monocots is quite different from that of dicots, MADS-box genes have been analyzed in monocots such as rice [8,9], maize [10,11], wheat [12], *Lolium temulentum* [13] and perennial ryegrass [14]. The ABC model could be also

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applicable to monocots by the results from two different species, maize and rice [15]. Maize gene *Silky1* [16] and rice gene *SUPERWOMAN1* (*SPW1*) [17], orthologues of *AP3*, specify the identity of stamens and lodicules; the latter organs correspond to the petals of *Arabidopsis*. The functions of *AG* gene have diversified and become partially redundant as a result of gene duplication in monocots. In maize, they may be separated with two genes, *ZMM2* for organ identity and *ZAG1* for floral meristem [18]. In rice, *OsMADS3* and *OsMADS58* genes also shared these functions [19]. Stamens in *silky1*, *spw1* and *osmads3* mutants and *OsMADS58* RNAi plants were homeotically transformed into other floral organs or changed morphologically by suppression of those transcription factors, in each case resulting in male sterility [16,17,19].

RNAi technology was applied to suppress *AG* in *Arabidopsis* [20] and *SPW1* in rice [21], but the frequencies of loss-of-function phenotype were low in rice [21]. Many plant transcription factors are members of large families. When there are functionally redundant transcription factors, suppression of one of these transcription factors is often less effective to induce loss-of-function phenotype. Chimeric REpressor gene-Silencing Technology (CRES-T) has been developed as a novel gene silencing method for transcription factors [22]. In this method, a target transcription factor is fused with the modified EAR-like motif repression domain (SRDX) to produce a chimeric repressor of the target transcription factor. When such a chimeric repressor is expressed in *Arabidopsis*, it can dominantly suppress the expression of target genes of the transcription factor and induce loss-of-function phenotype at high efficiency, even in the presence of functionally redundant transcription factors [22]. Thus, CRES-T is a powerful tool to induce loss-of-function phenotype. In addition, it is possible to apply CRES-T to plants with limited genome information because transcription factors are highly conserved between different species [23]. For example, chimeric repressor of *Arabidopsis* TCP3 induced similar phenotypes very effectively in torenia [24], chrysanthemum [24] and rose [25] without any change in the vector construct. The information has been collectively stored in the Fiore DB database [26]. CRES-T has also been applied to *AP3*, *AG* and *SPW1* to create sterile phenotypes [27]. Transgenic plants expressing each chimeric repressor of *AP3* and *AG* in *Arabidopsis* and *SPW1* in rice exhibited loss-of-function phenotype similar to mutant of the transcription factors themselves with high frequency [27]. Therefore, the chimeric repressors would be one of the useful tools to create male-sterile plants.

Tall fescue (*Festuca arundinacea* Schreb.) is a major cool-season perennial grass species that is widely used for both forage pastures and turf. Because of its agronomic importance, *Agrobacterium*-mediated transformation systems have been developed to improve its agronomic traits [28–30]. Since tall fescue is anemophilous, male sterility is strongly desired for transgenic tall fescue to prevent pollen dispersal. If the chimeric repressors can induce male sterility in tall fescue, they would be effective tools for practical application of transgenic plants in this species. For this purpose, we first introduced three chimeric repressors of rice *AG* orthologues, *Os12g0207000*, *Os01g0886200* and *OsMADS58*, into rice for evaluation of their abilities to induce male sterility and found that the chimeric repressor of *OsMADS58* was most suitable for our purpose. Next, we introduced two chimeric repressors of *SPW1* and *OsMADS58* into tall fescue to create male-sterile plants. In this study, we report on the alteration of floret traits using CRES-T.

2. Materials and methods

2.1. Phylogenetic analysis

The phylogenetic relationship of *AP3*- and *AG*-related genes was analyzed with ClustalW program. Bootstrap values were calculated from 1000 trials. Accession numbers of each gene or amino acid can be found in the GenBank/EMBL database: AtAP3 (P35632), *FaEST* (DT690242), *HvAP3* (AY541065), *TaAP3* (AB107993), *OsSPW1* (AK069317), *SbAP3* (XM_002438958), *ZmSilky1* (NM_001111481), *Os12g0207000* (AK070425), *OsMADS3* (L37528), *ZmAG1* (NM_001111851), *OsMADS58* (AK111723), *FpEST* (GO793260), *TaMADS* (BT008957), *HvAG2* (AF486649), AtAG (P17839), AtSHP2 (Q5XXG9), AtSHP1 (Q5XXJ3), *AtSTK* (NM_117064) and *Os01g0886200* (AK070958).

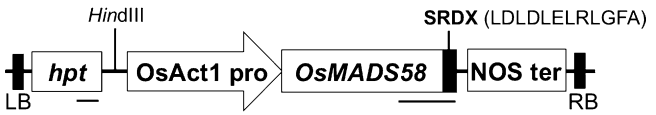

Fig. 1. Schematic representation of the chimeric repressor of *OsMADS58*. LB, left border; RB, right border; *hpt*, hygromycin phosphotransferase gene; OsACT1 pro, promoter of the rice *actin1* gene; *OsMADS58*, coding region of the *OsMADS58* gene without the stop codon; SRDX, repression domain isolated from the *SUPERMAN* gene of *Arabidopsis*; NOS ter, terminator of the nopaline synthase gene. The coding regions of the *Os12g0207000*, *Os01g0886200* and *SPW1* genes, without the stop codon, were each fused with SRDX sequence. The probes for Southern blot analysis are indicated by underlines.

2.2. Agrobacterium strain and vector constructs

The coding regions of the *SPW1* (AK069317), *Os12g0207000* (AK070425), *Os01g0886200* (AK070958) and *OsMADS58* (AK111723) genes, without the stop codon, were each fused with the SRDX sequence (LDLDLELRLGFA), a modified repression domain derived from *SUPERMAN* of *Arabidopsis* [22] and driven by the rice *actin1* promoter [31] (Fig. 1). These constructs (*SPW1SRDX*, *Os12g0207000SRDX*, *Os01g0886200SRDX* and *OsMADS58SRDX*) were transferred into the destination vector pBCKH carrying the hygromycin phosphotransferase gene (*hpt*) [27] by using the Gateway LR clonase reaction (Invitrogen, Carlsbad, CA, USA). These binary vectors were introduced into *Agrobacterium tumefaciens* strain EHA105 by electroporation (BioRad, Hercules, CA, USA).

2.3. Plant materials and genetic transformation

Agrobacterium-mediated transformation of rice cultivar 'Nipponbare' was performed as described [32]. Regenerated rice was grown in soil under natural sunlight in a greenhouse at 22–32 °C. The procedure for genetic transformation of tall fescue turf-type cultivar 'Tomahawk' was as described previously [33,34]. Regenerated tall fescue were vernalized in a cold room at 4 °C for 8 weeks and then transferred to a greenhouse at 23 °C. Pollen were stained with 1% carmine in 45% acetic acid and observed under a light microscope.

2.4. Southern blot analysis

Genomic DNA was extracted from leaf tissues by using the cetyltrimethylammonium bromide method [35] with some minor modifications. Genomic DNA (15 μg) was digested with *Hin*dIII (Fig. 1), fractionated in a 0.8% agarose gel at 30 V for 12 h, and blotted onto a positively charged nylon membrane (Roche, Mannheim, Germany). The gene-specific and *hpt* probes were amplified from each binary vector by using the PCR DIG probe synthesis kit (Roche) (Fig. 1). PCR was performed by using the following primers: SPW1-F (5′-CGTACGAGACTCTGCAGCAGGA-3′) and SRDX-R (5′-CAAACGGAGTTCTAGATCCA-3′) for *SPW1*, OsAG-F (5′-AAGGCCTAGGAAAGATTAGA-3′) and SRDX-R for *OsMADS58*, and hpt-F (5′-CGAAGAATCTCGTGCTTTCA-3′) and hpt-R (5′-TCCATCACAGTTTGCCAGTG-3′) for *hpt*. Southern blot analysis was carried out as described in the DIG manual (Roche).