Protocol for Agrobacterium-mediated transformation of tall fescue and future perspective on the application of genome editing

Tadashi Takamizo1, Hiroko Sato2,*

1 Institute of Livestock and Grassland Science, National Agriculture and Food Research Organization, Nasushiobara, Tochigi 329-2793, Japan; 2 Hokkaido Agricultural Research Center, National Agriculture and Food Research Organization, Sapporo, Hokkaido 062-8555, Japan
* E-mail: s.hiroko@affrc.go.jp  Tel: +81-11-857-9272  Fax: +81-11-859-2178

Received October 31, 2019; accepted March 9, 2020 (Edited by Y. Tabei)

Abstract  Tall fescue (Festuca arundinacea Schreb.) is a major cool-season perennial grass grown for forage and turf. We have obtained transgenic tall fescue by Agrobacterium-mediated transformation to improve agronomically important traits. In our protocol, we use embryogenic calli derived from not only mature seeds but also shoot tips. Although tall fescue cultivars consist of various genotypes with different genetic variation, we can produce transgenic plants at any time with calli induced from shoot tips of in vitro-maintained responsive genotypes. When the hygromycin phosphotransferase gene is used as a selectable marker, transformants are selected by incubation with 100 mg l−1 hygromycin in both selection and regeneration media. Since tall fescue is an anemophilous species, the cultivation of transgenic plants poses the risk of transgenic pollen flow. Recently, it has been reported that genome-edited plants without the integration of foreign DNA fragments can be produced by an Agrobacterium-mediated transient gene expression system. We hope that our protocol will contribute to production of transgene-free genome-edited tall fescue.

Key words:  Agrobacterium, genome editing, tall fescue, transformation.

Introduction  Tall fescue (Festuca arundinacea Schreb.) is widely used for forage and turf purposes in temperate regions worldwide. It is valued for its tolerance to various environmental stresses, its high persistence, and its adaptability to a wide range of soils. Since tall fescue is an allohexaploid (2n = 6x = 42) outcrossing species with self-incompatibility, conventional breeding based on selecting observable phenotypes is time-consuming, and thus genetic improvement is slow. Some transformation methods have been developed to facilitate genetic improvement and to complement conventional breeding in tall fescue. Since Agrobacterium tumefaciens (Rhizobium radiobacter) infects only dicot species in nature, monocot species were originally transformed only by direct gene transfer methods (Potrykus 1990). The first transgenic tall fescue was produced by polyethylene glycol- or electroporation-mediated direct gene transfer to protoplasts (Ha et al. 1992; Wang et al. 1992). These methods require plant regeneration from protoplasts, but many plants, including tall fescue, are recalcitrant to that. To generate transgenic plants from tall fescue, particle bombardment was applied to embryogenic calli or suspension-cultured cells (Cho et al. 2000; Spangenberg et al. 1995).

In 1994, Agrobacterium-mediated transformation of rice (Oryza sativa L.) was achieved by the inclusion of acetosyringone in the culture medium to mimic the phenolic substances produced by host plants that enable Agrobacterium infection (Hiei et al. 1994). Since then, many monocot species, including maize (Zea mays L.; Ishida et al. 1996), wheat (Triticum aestivum L.; Cheng et al. 1997), and barley (Hordeum vulgare L.; Trifonova et al. 2001), have been transformed by Agrobacterium. The first Agrobacterium-transformed tall fescue was generated by Bettany et al. (2003), who used embryogenic suspension-cultured cells as the target tissue. Dong and Qu (2005) and Wang and Ge (2005) used more convenient embryogenic calli derived from mature seeds as described in this protocol. Gao et al. (2008) detected GUS activity in leaves of 53% of Agrobacterium-
transformed lines but only 20% of bombarded lines, and concluded that the *Agrobacterium* method is superior.

Since tall fescue is a genetically heterogeneous species, cultivars consist of various genotypes responding differently in callus formation and plant regeneration (Takamizo et al. 1994). Therefore, it is impossible to obtain transgenic plants uniformly from all genotypes. In addition, the transformation efficiency also varies with genotypes. In prolonged callus culture, the risk of somaclonal variation increases, and a responsive genotype derived from a mature seed may lose its regeneration ability. If responsive genotypes can be preserved in vitro, we can produce transgenic plants at any time with calli induced from their shoot tips. This tissue culture system has been established in Italian ryegrass (*Lolium multiflorum* Lam.) transformation by particle bombardment (Takahashi et al. 2002, 2004). Similarly, transgenic forage-type perennial ryegrass (*Lolium perenne* L.) has been generated by *Agrobacterium*-mediated transformation (Sato and Takamizo 2006). When we introduced the binary vector pIG121Hm carrying the hygromycin phosphotransferase (*hpt*) gene as a selectable marker into the tall fescue cultivar ‘Tomahawk’ according to Sato and Takamizo (2006), hygromycin-resistant plants were obtained from 9 of 50 genotypes and the average transformation efficiency (the number of hygromycin-resistant plants/the number of calli infected with *Agrobacterium*) was 4.4% (1.5–7.5%) (unpublished data). Here, we describe our current protocol with some minor modifications of Sato and Takamizo (2006) using embryogenic calli induced from shoot tips of in vitro-maintained tall fescue plants.

### Protocol for *Agrobacterium*-mediated transformation of tall fescue

#### Media components

**Callus induction**, **co-cultivation**, and **selection media** are based on MS salts (Murashige and Skoog plant salt mixture: FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) and vitamins (Murashige and Skoog 1962), 30 g l\(^{-1}\) sucrose, 1 g l\(^{-1}\) casein hydrolysate, and 2 g l\(^{-1}\) Gelrite (FUJIFILM Wako). In addition, the callus induction medium contains 5 mg l\(^{-1}\) 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.2 mg l\(^{-1}\) 6-benzylaminopurine (BAP); the co-cultivation medium contains 10 g l\(^{-1}\) glucose, 2 mg l\(^{-1}\) 2,4-D, and 100 µM acetosyringone; and the selection medium contains 2 mg l\(^{-1}\) 2,4-D, 0.2 mg l\(^{-1}\) BAP, 100 mg l\(^{-1}\) hygromycin, and 250 mg l\(^{-1}\) carbenicillin. The regeneration medium is based on MS medium containing 30 g l\(^{-1}\) sucrose, 0.2 mg l\(^{-1}\) kinetin, and 4 g l\(^{-1}\) Gelrite. Only the co-cultivation medium is adjusted to pH 5.2, and all other media are adjusted to pH 5.8 prior to being autoclaved at 121°C for 20 min.

#### Callus induction

Mature seeds of the forage-type cultivar ‘Nanryo’ and the turf-type cultivar ‘Tomahawk’ are stirred in 64% sulfuric acid for 30 min to remove palea and lemma and then rinsed in distilled water several times. They are then stirred in 70% ethanol for 1 min, then in sodium hypochlorite containing 5% available chlorine for 20 min, and rinsed in sterile water five times. Sterilized mature seeds are germinated on regeneration medium without kinetin (hormone-free) and plants are maintained in vitro under a 8/16 h (day/night) photoperiod (4000 lx fluorescent light). Shoot tips are isolated from plants with a scalpel under a microscope (Figure 1A) and placed on callus induction medium in the dark at 25°C. Similarly, embryogenic calli induced from sterilized mature seeds can also be used in this protocol (Figure 1B). Induced calli are transferred to fresh medium every 4 weeks. Embryogenic calli derived from shoot tips isolated from a single plant are grown separately as individual genotypes. As negative controls, wild-type plants are regenerated from non-transgenic calli of the same genotypes used for genetic transformation because genetic variations exist among genotypes of tall fescue.

#### Co-cultivation of calli with *Agrobacterium*

*Agrobacterium tumefaciens* strain EHA105 carrying a binary vector is grown in YEP medium (10 g l\(^{-1}\) yeast extract, 10 g l\(^{-1}\) peptone, 5 g l\(^{-1}\) NaCl, 2 mM MgCl\(_2\), pH 7.2) supplemented with appropriate antibiotics with agitation in the dark at 28°C for 20 h. The *Agrobacterium* suspension is collected by centrifugation (\(1500\times g\)) for 10 min and resuspended at an OD\(_{600}\) of 0.1 in AAM medium (Hiei et al. 1994) with concentrations of AA salts and amino acids as described by Toki et al. (2006). Calli are transferred onto a stainless pot (Cell dissociation sieve-tissue grinder kit: Sigma-Aldrich, St. Louis, MO, USA) with nylon mesh (305 mesh) and immersed in the *Agrobacterium* suspension for 5 min (Figure 1C). During this step, calli are broken into small pieces using forceps. Excess suspension is removed by blotting on sterile filter paper. The infected calli are co-cultivated on sterile filter paper moistened with AAM medium on co-cultivation medium in the dark at 25°C for 4 days. The calli are then rinsed with 250 mg l\(^{-1}\) carbenicillin in sterile water three times or more to eliminate the *Agrobacterium*.

#### Selection and regeneration

When the *hpt* gene under the control of the cauliflower mosaic virus 35S promoter is used as a selectable marker, the infected calli are cultured on selection medium in the dark at 25°C. After 4 weeks, hygromycin-resistant calli are transferred to fresh selection medium (Figure 1D). After 8 weeks in total, hygromycin-resistant calli are placed on regeneration medium supplemented with...
100 mg l\(^{-1}\) hygromycin and 250 mg l\(^{-1}\) carbenicillin under short-day conditions (8 h light/16 h dark) at 25°C (Figure 1E). Although the hpt gene is the most frequently used selectable marker in tall fescue, herbicide-resistance genes such as bar in combination with bialaphos or phosphinothricin (Gao et al. 2008; Long et al. 2011) and a mutated rice acetolactate synthase gene together with pyriminobac (Sato et al. 2013) can also be used.

Regenerated plants are transferred to soil and grown in a greenhouse at 20–23°C (Figure 1F). They are vernalized to induce flowering in a cold room under short-day conditions (8 h light/16 h dark) at 4°C for 8 weeks and then returned to the greenhouse. Their spikelets are crossed with those of other wild-type genotypes within a paper bag. Because of the risk of transgenic pollen flow, the pollen of transgenic plants is applied to the florets of a cytoplasmic male sterile (CMS) tall fescue carrying the cytoplasm of Italian ryegrass (Iriyama et al. 2009) to result in CMS T\(_1\) seeds, thereby preventing the dispersal of transgenic pollen, because no restorer gene has so far been found.

**Future perspective on tall fescue transformation toward genome editing**

To improve tall fescue by genetic transformation, researchers have introduced agronomically important genes involved in forage digestibility (Chen et al. 2003, 2004; Sato et al. 2018); tolerance to cold (Hu et al. 2005), drought (Zhao et al. 2007a), heat (Wang et al. 2017), salt (Ma et al. 2014; Zhao et al. 2007b), and a wide range of abiotic stresses (Kim et al. 2010, 2012; Lee et al. 2007; Lee et al. 2012); disease resistance (Dong et al. 2007, 2008; Zhou et al. 2016a, b); and herbicide resistance (Sato et al. 2009). However, its wind-pollinating and invasive characteristics hamper practical commercialization, even in the USA, where genetically modified crops are widespread.

Genome editing, which can efficiently change just the target gene, is now an important technique for use with all organisms. Genome editing experiments now favor clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein 9 (Cas9; Jinek et al. 2012) because of its ease of handling compared to other techniques based on ZFN (zinc finger nuclease) or TALEN (transcription activator-like effector nuclease). Its application is attractive because genome-edited plants without foreign genes such as selective marker genes can be acquired due to the segregation by crossing with wild-type plants. However, the broader application of plasmid-mediated CRISPR/Cas9 to the life sciences, especially medicine, is limited by off-target effects (Zhang et al. 2015) such as unwanted induction of mutations into the genome and possible regulations for genetically modified organisms.

DNA-free genome editing, such as the introduction of CRISPR/Cas9 ribonucleoprotein, has been developed, and the resultant organisms are far less likely to be regulated, as no DNA is introduced (Metje-Sprink et al. 2019). Because it is impossible to introduce
ribonucleoprotein into plant cells with *Agrobacterium*, direct gene transfer methods were chosen at first. Namely, CRISPR/Cas9 ribonucleoprotein without plasmid DNA was introduced by direct gene transfer to protoplasts into *Arabidopsis thaliana* L., coyote tobacco (*Nicotiana attenuata* L.), lettuce (*Lactuca sativa* L.) and rice (*Oryza sativa* L.) by particle bombardment into immature maize embryos (Svitashhev et al. 2016). About the transformation of tall fescue, direct gene transfer by both protoplast (Ha et al. 1992; Wang et al. 1992) and particle bombardment methods (Cho et al. 2000; Spangenberg et al. 1995) is available. Their transformation efficiencies do not differ much, but both methods have merit and demerit. Although plant regeneration from protoplasts in monocots is only possible from embryogenic suspension-cultured cells which requires much time and labor to establish, plant regeneration from protoplasts has less risk of chimerism. On the other hand, particle bombarded tissues sometimes produce chimeric plants with transformed and non-transformed sectors (Fitch et al. 1990). If CRISPR/Cas9 ribonucleoprotein is not introduced into germ cells that differentiate into male or female gametes, the modified target gene cannot be inherited into the progeny. However, this problem will be solved to introduce CRISPR/Cas9 ribonucleoprotein into shoot apical meristems of mature embryos using in planta particle bombardment method as described by Hamada et al. (2017).

Recently, it has been shown that transient CRISPR/Cas9 gene expression using *Agrobacterium*-mediated transformation can just create genome-edited-plants in tobacco (Chen et al. 2018), potato (*Solanum tuberosum* L.) and tomato (*Solanum lycopersicum* L.) (Veillet et al. 2019). The created genome-edited plants do not have to be backcrossed with wild-type plants to eliminate foreign DNA fragments and can be readily used as breeding material without changing original genetic background. It is essential for forage and turf grasses including outcrossing or apomictic species. Those methods use leaf, cotyledon, stem and petiole tissues as explants (Chen et al. 2018; Veillet et al. 2019). As far as we know, transient CRISPR/Cas9 gene expression using embryogenic calli has not yet been reported. However, we expect our protocol for *Agrobacterium*-mediated transformation will be applied to produce transgene-free genome-edited tall fescue in near future.

**References**

Bettnany AJE, Dalton SJ, Timms E, Manderyck B, Dhanoa MS, Morris P (2003) *Agrobacterium tumefaciens*-mediated transformation of *Festuca arundinacea* (Schreb.) and *Lolium multiflorum* (Lam.). Plant Cell Rep 21: 437–444

Chen L, Auh CK, Dowling P, Bell J, Chen F, Hopkins A, Dixon RA, Wang ZY (2003) Improved forage digestibility of tall fescue (*Festuca arundinacea*) by transgenic down-regulation of cinnamyl alcohol dehydrogenase. *Plant Biotechnol J* 1: 437–449

Chen L, Auh CK, Dowling P, Bell J, Lehmann D, Wang ZY (2004) Transgenic down-regulation of caffeic acid O-methyltransferase (COMT) led to improved digestibility in tall fescue (*Festuca arundinacea*). *Func Plant Biol* 31: 235–245

Chen L, Li W, Katin-Grazzini L, Ding J, Gu X, Li Y, Gu T, Wang R, Lin X, Deng Z, et al. (2018) A method for the production and expedient screening of CRISPR/Cas9-mediated non-transgenic mutant plants. *Hortic Res* 5: 13

Cheng M, Fry JE, Pang S, Zhou H, Hironaka CM, Duncan DR, Conner TW, Wan Y (1997) Genetic transformation of wheat mediated by *Agrobacterium tumefaciens*. *Plant Physiol* 115: 971–980

Cho MJ, Ha CD, Lemaux PG (2000) Production of transgenic tall fescue and red fescue plants by particle bombardment of mature seed-derived highly regenerative tissues. *Plant Cell Rep* 19: 1084–1089

Dong S, Qu R (2005) High efficiency transformation of tall fescue with *Agrobacterium tumefaciens*. *Plant Sci* 168: 1453–1458

Dong S, Shew HD, Tredway LP, Lu J, Sivamani E, Miller ES, Qu R (2008) Expression of the bacteriophage T4 lysozyme gene in tall fescue confers resistance to gray leaf spot and brown patch diseases. *Transgenic Res* 17: 47–57

Dong S, Tredway LP, Shew HD, Wang GL, Sivamani E, Qu R (2007) Resistance of transgenic tall fescue to two major fungal diseases. *Plant Sci* 173: 501–509

Fitch MMM, Manshardt RM, Gonsalves D, Slightom JL, Sanford JC (1990) Stable transformation of papaya via microprojectile bombardment. *Plant Cell Rep* 9: 189–194

Gao C, Long D, Lenk I, Nielsen KK (2008) Comparative analysis of transgenic tall fescue (*Festuca arundinacea* Schreb.) plants obtained by *Agrobacterium*-mediated transformation and particle bombardment. *Plant Cell Rep* 27: 1601–1609

Ha SB, Wu FS, Thorne TK (1992) Transgenic turf-type tall fescue (*Festuca arundinacea* Schreb.) plants regenerated from protoplasts. *Plant Cell Rep* 11: 601–604

Hamada H, Linghu Q, Nagira Y, Miki R, Takaoka N, Imai R (2017) An in planta biolistic method for stable wheat transformation. *Sci Rep* 7: 11443

Hiei Y, Ohta S, Komari T, Kumashiro T (1994) Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. *Plant J* 6: 271–282

Hu Y, Jia W, Wang J, Zhang Y, Yang L, Lin Z (2005) Transgenic tall fescue containing the *Agrobacterium tumefaciens ipt* gene shows enhanced cold tolerance. *Plant Cell Rep* 23: 705–709

Iriyama Y, Tachibana T, Fujimori M, Arakawa A, Komatsu T, Takamizo T (2009) Characteristics investigation of male-sterile tall fescue “MST1” considering biodiversity. *Jpn Soc Reveget Tech* (Nihonryokukakougaku Kaishi) 35: 107–110

Ishida Y, Saito H, Ohta S, Hiei Y, Komari T, Kumashiro T (1996) High efficiency transformation of maize (*Zea mays* L.) mediated by *Agrobacterium tumefaciens*. *Nat Biotechnol* 14: 745–750

Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 337: 816–821

Kim KH, Alam I, Kim YG, Sharmin SA, Lee KW, Lee SH, Lee BH (2012) Overexpression of a chloroplastic-localized small heat shock protein OsHSP26 confers enhanced tolerance against oxidative and heat stresses in tall fescue. *Biotechnol Lett* 34: 371–377
Kim KH, Alam I, Lee KW, Sharmin SA, Kwak SS, Lee SY, Lee BH (2010) Enhanced tolerance of transgenic tall fescue plants overexpressing 2-Cys peroxiredoxin against methyl viologen and heat stresses. Biotechnol Lett 32: 571–576
Lee KW, Choi GI, Kim KY, Ji HJ, Park HS, Kim YG, Lee BH, Lee SH (2012) Transgenic expression of MsHsp23 confers enhanced tolerance to abiotic stresses in tall fescue. Asian–Aust J Anim Sci 25: 818–823
Lee SH, AhSan N, Lee KW, Kim DH, Lee DG, Kwak SS, Kwon SY, Kim TH, Lee BH (2007) Simultaneous overexpression of both CuZn superoxide dismutase and ascorbate peroxidase in transgenic tall fescue plants confers increased tolerance to a wide range of abiotic stresses. J Plant Physiol 164: 1626–1638
Long D, Wu X, Yang Z, Lenk I, Nielsen KK, Gao C (2011) Comparison of three selectable marker genes for transformation of tall fescue (Festuca arundinacea Schreb.) plants by particle bombardment. In Vitro Cell Dev Biol Plant 47: 658–666
Ma DM, Xu WR, Li HW, Jin FX, Guo LN, Wang J, Dai HJ, Xu X (2014) Co-expression of the Arabidopsis SOS genes enhances salt tolerance in transgenic tall fescue (Festuca arundinacea Schreb.). Protoplasma 251: 219–231
Metje-Sprink J, Menz J, Modrzejewski D, Sprink T (2019) DNA-free genome editing: Past, present and future. Front Plant Sci 9: 1957
Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15: 473–497
Potrykus I (1990) Gene transfer to cereals: An assessment. Bio. Technol 8: 535–542
Sato H, Sakamoto S, Mitsuda N, Ohme-Takagi M, Takamizo T (2018) Improvement of cell wall digestibility in tall fescue by Oryza sativa SECONDARY WALL NAC DOMAIN PROTEIN2 chimeric repressor. Mol Breed 38: 36
Sato H, Shimizu T, Kawai K, Kaku K, Arakawa A, Tachibana T, Takamizo T (2013) Herbicide-resistant tall fescue with cytoplasmic male sterility selected by a mutated rice acetolactate synthase gene. Mol Sci 53: 201–207
Sato H, Shimizu T, Kawai K, Kaku K, Takamizo T (2009) Conferrered resistance to an acetolactate synthase-inhibiting herbicide in transgenic tall fescue (Festuca arundinacea Schreb.). J Plant Physiol 162: 103–113
Woo JW, Kim J, Kwon SI, Corvalán C, Cho SW, Kim H, Kim SG, Kim ST, Choe S, Kim JS (2015) DNA-free genome editing in plants with preassembled CRISPR-Cas9 ribonucleoproteins. Nat Biotechnol 33: 1162–1165
Zhang XH, Tee LY, Wang XG, Huang QS, Yang SH (2015) Off-target effects in CRISPR/Cas9-mediated genome engineering. Mol Ther Nucleic Acids 4: e264
Zhao JH, Ren W, Zhi D, Wang L, Xia G (2007a) Arabidopsis DREB1A/CBF3 bestowed transgenic tall fescue increased tolerance to drought stress. Plant Cell Rep 26: 1521–1528
Zhao J, Zhi D, Xue Z, Liu H, Xia G (2007b) Enhanced salt tolerance of transgenic progeny of tall fescue (Festuca arundinacea) expressing a vacuolar Na’/H’ antiporter gene from Arabidopsis. J Plant Physiol 164: 1377–1383
Zhou B, Bailey A,Niblett CL, Qu R (2016a) Control of brown patch (Rhizoctonia solani) in tall fescue (Festuca arundinacea Schreb.) by host induced gene silencing. Plant Cell Rep 35: 791–802
Zhou B, Luo H, Qu R (2016b) Expression of the shrimp antimicrobial peptide penaeidin 4-1 confers resistance against brown patch disease in tall fescue. Plant Cell Tissue Organ Cult 125: 599–603

Takahashi W, Oishi H, Ebina M, Takamizo T, Komatsu T (2002) Production of transgenic Italian ryegrass (Lolium multiflorum Lam.) via microprojectile bombardment of embryogenic calli. Plant Biotechnol 19: 241–249
Takamizo T, Fukase N, Saruwatari Y, Sugino K (1994) Genetic variation in plant regeneration from callus culture of tall fescue (Festuca arundinacea Schreb.) cultivars. Inpn Agric Res Q 28: 200–205
Tok i S, Hara N, Ono K, Onodera H, Tagiri A, Oka S, Tanaka H (2006) Early infection of scutellum tissue with Agrobacterium allows high-speed transformation of rice. Plant J 47: 969–976
Tritonova A, Madsen S, Olesen A (2001) Agrobacterium-mediated transgene delivery and integration into barley under a range of vitro culture conditions. Plant Sci 161: 871–880
Veillet F, Perrot L, Chauvin L, Kermarrec MP, Guyon-Debast A, Chauvin JE, Nogué F, Mazier M (2019) Transgene-free genome editing in tomato and potato plants using Agrobacterium-mediated delivery of a CRISPR/ Cas9 cytidine base editor. Int J Mol Sci 20: 402
Wang X, Huang W, Liu J, Yang Z, Huang B (2017) Molecular regulation and physiological functions of a novel FaHsfA2c cloned from tall fescue conferring plant tolerance to heat stress. Plant Biotechnol J 15: 237–248
Wang ZY, Takamizo T, Iglesias VA, Usisky M, Nagel J, Potrykus I, Spangenberg G (1992) Transgenic plants of tall fescue (Festuca arundinacea Schreb.) obtained by direct gene transfer to protoplasts. Bio/Technol 10: 691–696
Wang ZY, Ge Y (2005) Agrobacterium-mediated high efficiency transformation of tall fescue (Festuca arundinacea). J Plant Physiol 162: 103–113
Woo JW, Kim J, Kwon SI, Corvalán C, Cho SW, Kim H, Kim SG, Kim ST, Choe S, Kim JS (2015) DNA-free genome editing in plants with preassembled CRISPR-Cas9 ribonucleoproteins. Nat Biotechnol 33: 1162–1165
Zhang XH, Tee LY, Wang XG, Huang QS, Yang SH (2015) Off-target effects in CRISPR/Cas9-mediated genome engineering. Mol Ther Nucleic Acids 4: e264
Zhao J, Ren W, Zhi D, Wang L, Xia G (2007a) Arabidopsis DREB1A/CFB3 bestowed transgenic tall fescue increased tolerance to drought stress. Plant Cell Rep 26: 1521–1528
Zhao J, Zhi D, Xue Z, Liu H, Xia G (2007b) Enhanced salt tolerance of transgenic progeny of tall fescue (Festuca arundinacea) expressing a vacuolar Na’/H’ antiporter gene from Arabidopsis. J Plant Physiol 164: 1377–1383
Zhou B, Bailey A, Niblett CL, Qu R (2016a) Control of brown patch (Rhizoctonia solani) in tall fescue (Festuca arundinacea Schreb.) by host induced gene silencing. Plant Cell Rep 35: 791–802
Zhou B, Luo H, Qu R (2016b) Expression of the shrimp antimicrobial peptide penaeidin 4-1 confers resistance against brown patch disease in tall fescue. Plant Cell Tissue Organ Cult 125: 599–603