Evaluation of transgenic tall fescue (*Festuca arundinacea* Schreb.) improved for important forage and turf traits

（牧草および芝草の重要形質を改良したトールフェスク形質転換体の評価）

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Abbreviations

2,4-D	2,4-dichlorophenoxyacetic acid
ADF	acid detergent fiber
AG	AGAMOUS
AIR	alcohol-insoluble residue
ALS	acetolactate synthase
AP3	APETALA3
APHIS	Animal and Plant Health Inspection Service
BC	backcross
bc	brittle culm
BS	bispyribac-sodium
Cas9	CRISPR-associated protein 9
CAD	cinnamyl alcohol dehydrogenase
CMS	cytoplasmic male-sterile
COMT	caffeic acid *O*-methyltransferase
CRISPR	clustered regularly interspaced short palindromic repeats
CTAB	cetyltrimethylammonium bromide
DIG	digoxigenin
EAR	ethylene-responsive element binding factor-associated amphiphilic repression
EST	expressed sequence tag
FSCJ	Food Safety Commission of Japan
FW	fresh weight
GM	genetically modified
HR	herbicide-resistant
hpt	hygromycin phosphotransferase
LB	left border

MOE	Ministry of the Environment
NAC	NAM, ATAF1/2 and CUC2
NOS	nopaline synthase
NPBT	new plant breeding techniques
NST	NAC SECONDARY WALL THICKENING PROMOTING FACTOR
PC	pyrimidinylcarboxylate
PM	pyriminobac
PS	pyrithiobac-sodium
RB	right border
SD	standard deviation
SE	standard error
SPW1	SUPERWOMAN1
SRDX	modified EAR-like motif repression domain
SWN	SECONDARY WALL NAC DOMAIN PROTEIN
Syn	synthetic generation
TF	transcription factor
USDA	U.S. Department of Agriculture
VND	VASCULAR-RELATED NAC DOMAIN

Chapter 1: General introduction

1.1: Important roles of cool-season grasses in grasslands

Forage grasslands, including rangelands, pasturelands and croplands sown with pasture and fodder crops, covered approximately 3.5 billion hectares in 2000, representing 26% of the world land area and 70% of the world agricultural area, and containing 20% of the world's soil carbon stocks (FAO 2010). Grasslands provide feed to produce livestock products (e.g., milk, meat and wool) from ruminants such as cattle, goats and sheep; they also play important environmental roles by protecting water resources, preserving biological diversity and mitigating climate change through carbon storage (FAO 2010). Total feed biomass consumed by ruminants was approximately 3.7 billion tons in 2000, of which grasses comprised 62% (2.3 billion tons) (Herrero et al. 2013). Grasses are also used as amenity turf of athletic fields, golf courses and lawns and for roadside cover to provide a safe playing surface for sports and recreation and prevent soil erosion. Total world grass seed production in major countries was estimated to be approximately 0.68 million tons in 2003 (Wong 2005). Forage and turf grasses boast one of the largest seed markets in the world. Sustainable grasslands are important not only for livestock production but also for the environment and landscape.

In comparison with warm-season grasses, which are better adapted to high temperatures, cool-season grasses have several advantages such as markedly superior nutritive quality, resulting in benefits for livestock production, and higher yields during cooler seasons (Norton et al. 2016). In temperate regions including Japan, cool-season grasses are predominant and are grown primarily for pasture, silage and hay; they are the main source of energy for ruminants. Major cool-season grass species include Italian ryegrass (*Lolium multiflorum* Lam.), meadow fescue (*Festuca pratensis* Huds.), orchardgrass (*Dactylis glomerata* L.), perennial ryegrass (*Lolium perenne* L.), tall fescue (*Festuca arundinacea* Schreb.) and timothy (*Phleum pratense* L.). Some slow-growing dwarf-type species such as creeping bentgrass (*Agrostis stolonifera* L.), Kentucky bluegrass (*Poa pratensis* L.), perennial ryegrass and tall fescue are important for turf and roadside cover.

1.2: Tall fescue

Tall fescue is cultivated in North and South America, North Africa, Europe and Asia. After ryegrasses, it was the second most widely produced grass seed worldwide in 2003 (Wong 2005). Native to Europe and North Africa, tall fescue is an allohexaploid (2n = 6*x* = 42) and contains three genomes: the P genome is derived from diploid *F. pratensis*, and the G_1 and G_2 genomes are from tetraploid *F. arundinacea* var. *glaucescens* (Humphreys et al. 1995; Sleper and West 1996). In Japan, tall fescue is grown for forage in warm regions where other cool-season grasses would not grow. It is valued for its tolerance to various environmental stresses and its high persistence. The cultivation of tall fescue is expected to increase in temperate regions due to global warming. Tall fescue is used for turf and roadside cover because of its adaptability to a wide range of soils. On the other hand, its forage digestibility and palatability are often lower than those of other cool-season grasses. Another disadvantage is that tall fescue is an anemophilous (wind-pollinated) species and produces large amounts of pollen containing allergenic proteins that cause hay fever in susceptible people.

The tall fescue cultivar 'Kentucky 31' was released in the USA in 1942 (Fergus and Buckner 1972), and was introduced into Japan during the 1960s and 1970s. In Japan, the cultivar became popular for forage, turf and roadside cover. Conventional breeding for forage use was carried out to increase its adaptability to environmental conditions in Japan. In 1972, two cultivars, 'Yamanami' and 'Hokuryo', were released by the Hokkaido National Agricultural Experiment Station. 'Nanryo' (1984) were bred at the Kyushu National Agricultural Experiment Station, and 'Ushibue' (2005) was released by the Kyushu Okinawa Agricultural Research Center. 'Southern cross' (1986) was bred by the Snow Brand Seed Company. The breeding objectives include increased forage digestibility, stress tolerance in summer, and seed production. To prevent pollen flow, 'MST1' was bred by the National Institute of Livestock and Grassland Science and the Snow Brand Seed Company, and was registered as a cytoplasmic male-sterile (CMS) cultivar for roadside cover in 2006 (Iriyama et al. 2009). There is no cultivar bred for turf in Japan; fescue grass seeds for turf and roadside cover are imported and were estimated to be 552.6 tons in 2017 (Yoshihara 2018). Tall fescue is closely related to *Lolium* species. *Festulolium* (intergeneric hybrids between *Lolium* and *Festuca* species) has been developed to combine

the forage quality of *Lolium* species with the environmental stress tolerance of *Festuca* species (Humphreys et al. 2003; Yamada et al. 2005).

1.3: Conventional breeding in cool-season grasses

Conventional breeding has enhanced agronomic performance in cool-season grasses including tall fescue by using the natural gene pool of varieties or ecotypes. Most grasses are outcrossing polyploid species with a high degree of self-incompatibility, which complicates trait inheritance (Talukder and Saha 2017). These reproductive characteristics and genetic complexity make it difficult to improve their agronomic traits. Each cultivar is a heterogeneous population with heterozygous genotypes maintained by crossing a variable number of selected individuals to avoid inbreeding depression. Conventional breeding on the basis of selecting observable phenotypes is time-consuming and uses complicated recurrent selection to accumulate favorable alleles in a population (Vogel and Pedersen 1993). The genetic improvement of forage grasses appears to be progressing more slowly than that of grain crops. For example, the average rate of genetic gain in dry matter yield is lower in forage grasses (3.6% per decade in Italian ryegrass, orchardgrass, perennial ryegrass and tall fescue) than in grain crops [13.5% per decade in barley (*Hordeum vulgare* L.), bread wheat (*Triticum aestivum* L.), maize (*Zea mays* L.) and oats (*Avena sativa* L.)] (Humphreys 1999).

By the end of the 21st century, the global average ground surface temperature is predicted to increase between 0.3°C and 4.8°C relative to 1986–2005 (IPCC 2014). Extreme temperatures and rainfalls, water shortages and changes in soil quality will limit plant growth, shift production seasons and change the patterns of diseases, pests, weeds, prices and incomes (Andjelkovic 2018; Rojas-Downing et al. 2018). Climate change will strongly affect pasture production and turf maintenance. Demand for livestock products is expected to grow strongly due to world population growth and income growth in developing countries (O'Mara 2012). On the other hand, agricultural area including grasslands has remained static since the early 1990s (World Bank Open Data 2018). For stable cultivation and high production in grasslands, it is essential to enhance agronomic performance by plant breeding more quickly and effectively.

1.4: Improvement of tall fescue by genetic transformation

Genetic transformation complements conventional breeding and facilitates plant improvement. It allows the direct introduction of agronomically useful genes from any organism into target plants and the down- or up-regulation of endogenous genes to generate desired phenotypes and novel genetic variants (Wang et al. 2009). In tall fescue, genetic transformation systems have been developed based on both particle bombardment (Cho et al. 2000; Spangenberg et al. 1995) and *Agrobacterium*-mediated transformation (Dong and Qu 2005; Lee et al. 2004; Wang and Ge 2005). The latter is superior to the former because it produces transgenic plants with low-copy transgene insertions, fewer rearrangements and stable transgene expression (Dai et al. 2001; Shou et al. 2004). *Agrobacterium*-mediated transformation is reportedly the preferred method to analyze transgene functions in tall fescue (Gao et al. 2008). Genetic transformation of tall fescue has been employed to improve forage digestibility (Chen et al. 2003, 2004); 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); and disease resistance (Dong et al. 2007, 2008; Zhou et al. 2016a, 2016b).

The adoption of genetically modified (GM) crops has increased crop yields and farmer profits (Brookes and Barfoot 2017b; Klümper and Qaim 2014) and decreased the environmental impacts associated with pesticide spraying and fuel use (Brookes and Barfoot 2017a). In 2016, GM crops including maize, soybean [*Glycine max* (L.) Merr.], cotton (*Gossypium hirsutum* L.) and oilseed rape (canola; *Brassica napus* L.) were cultivated on approximately 185.1 million hectares in 26 countries, which correspond to 12% of the world arable area (ISAAA 2016). Transgenic approaches have been used to improve cool-season grasses (Giri and Praveena 2015; Wang and Ge 2006), but the only deregulated transgenic forage crop is alfalfa (*Medicago sativa* L.) resistant to the herbicide glyphosate (Waltz 2011). Recently, the U.S. Department of Agriculture's (USDA) Animal and Plant Health Inspection Service (APHIS) concluded that "transgenic creeping bentgrass resistant to glyphosate is unlikely to pose a plant pest risk to agricultural crops or other plants in the USA" (USDA 2016, 2017). The deregulation was announced in 2017 (USDA 2017). Since major cool-season grasses are cross-pollinated and anemophilous species, risk assessment of transgenic grasses has focused on their

environmental and ecological impacts due to transgenic pollen flow; their commercialization has been limited by stringent and costly regulatory requirements (Wang and Brummer 2012).

1.5: Objectives and composition of this thesis

Conventional breeding of tall fescue is time-consuming, and its genetic improvement is slow because it is an outcrossing allohexaploid species with self-incompatibility. Genetic transformation of tall fescue has been developed to complement conventional breeding and facilitate genetic improvement (see Chapter 1.4). When a potentially useful gene is tested, in some cases the gene can be used directly, whereas in other cases the transgenic plants do not have the predicted phenotypes even though the gene function is known in the species of origin. In this study, transgenic tall fescue was produced with the objective of improving important forage and turf traits, forage digestibility, herbicide resistance and male sterility by using *Agrobacterium*-mediated transformation, and transgene function was evaluated by molecular and phenotypic analyses to use as breeding materials.

Because forage digestibility directly affects livestock performance, its improvement is an important target in breeding. Some *brown-midrib* mutants of maize, which produce reddish-brown pigmentation associated with lignified tissues, have reduced lignin content and increased digestibility due to mutations in cinnamyl alcohol dehydrogenase (CAD) or caffeic acid *O*-methyltransferase (COMT) in the lignin biosynthetic pathway (Halpin et al. 1998; Vignols et al. 1995). Down-regulation of these genes also led to improved digestibility in alfalfa (Baucher et al. 1999; Guo et al. 2001), tall fescue (Chen et al. 2003, 2004) and perennial ryegrass (Tu et al. 2010). These results indicate that forage digestibility is negatively correlated with lignin content. The lower forage digestibility in tall fescue is due in part to fiber-constituting sclerenchyma cells, which have high cellulose and lignin contents (Chen et al. 2002). Thus, sclerenchyma cells can be an important target to improve forage digestibility by genetic manipulation. In rice (*Oryza sativa* L.), the SECONDARY WALL NAC (NAM ATAF1/2 and CUC2) DOMAIN PROTEIN2 (OsSWN2) chimeric repressor under the control of the *OsSWN1* promoter reduces secondary cell wall thickening in sclerenchyma cells (Yoshida et al. 2013). In Chapter 2, the *OsSWN2* chimeric repressor was introduced into tall fescue to improve forage digestibility. In

turfgrass, herbicide resistance allows easier weed elimination while providing economic and environmental benefits by reducing production costs, herbicide spraying and fuel use (Green 2012). Pyrimidinylcarboxylates (PCs) inhibit acetolactate synthase (ALS; also known as acetohydroxyacid synthase) in the biosynthetic pathway leading to the branched-chain amino acids (Shimizu et al. 2002). ALS does not exist in mammals, and PCs are therefore thought to be less toxic to mammals than to plants. Transgenic plants resistant to PCs has not been reported in cool-season grasses. A rice *ALS* gene containing a single point mutation [*OsALS* (sm)] can confer resistance to the PC herbicide pyriminobac (PM) (Kawai et al. 2008). In Chapter 3, the *OsALS* (sm) gene was used to produce herbicide-resistant (HR) tall fescue. The cultivation of transgenic grasses poses the risk of transgenic pollen flow and consequent transgene introgression into sextually compatible wild species, endangering biological diversity. Male sterility is a possible approach to prevent the dispersal of transgenic pollen into the environment. When an agronomically useful gene and a male-sterile gene reside in the same construct, they will be introduced simultaneously, and pollen production by the transgenic plants will be inhibited. *SUPERWOMAN1* (*SPW1*) and *OsMADS58* specify the formation of stamens in rice (Nagasawa et al. 2003; Yamaguchi et al. 2006). Stamens in the loss-of function phenotypes of these genes were homeotically transformed into other floral organs, where they resulted in morphological changes that led to male sterility (Mitsuda et al. 2006; Nagasawa et al. 2003; Yamaguchi et al. 2006). In Chapter 4, two chimeric repressors of *SPW1* and *OsMADS58* were evaluated for their abilities to induce male sterility.

Because cultivars consist of various genotypes, with each genotype behaving differently in callus formation and plant regeneration, most genotypes used for genetic transformation are selected on the basis of their response in tissue culture rather than agronomic performance. For practical applications in outcrossing grasses, it is necessary to incorporate transgenes into non-transgenic elite genotypes with agronomically superior traits by backcrossing. Recently, cisgenesis and genome editing have been developed as new plant breeding techniques (NPBT) (Cardi 2016). Novel GM plants developed with NPBT are expected to have increased consumer acceptance compared with those produced using traditional genetic transformation. Finally, the strategies for breeding of tall fescue by transgenic approaches including NPBT are discussed.

Chapter 2: Improvement of forage digestibility by *Oryza sativa* SECONDARY WALL NAC DOMAIN2 chimeric repressor

2.1: Introduction

Forage digestibility improves livestock performance. Dry matter intake in ruminants is influenced by the rate of fiber digestion, the rate of passage through rumen, and the proportion of indigestible fiber (Mertens and Ely 1979). A 1% increase in neutral detergent fiber digestibility in forage crops including alfalfa, bread wheat, maize, sorghum [*Sorghum bicolor* (L.) Moench] and timothy is associated with a 0.17-kg increase in dry matter intake of dairy cows and a 0.25-kg increase in milk production (Oba and Allen 1999).

Fiber digestion depends on cell type. Parenchyma cells are rapidly and extensively digested owing to their small size and thin cell walls. In contrast, sclerenchyma cells are digested slowly and incompletely because of their thick secondary cell walls and low exposure of surface area to rumen microorganisms (Grabber et al. 1992). Secondary cell walls, composed mainly of cellulose, hemicellulose and lignin, are formed inside primary cell walls after cell expansion has ceased. They provide strength for water transport and mechanical support in vascular bundles and sclerenchyma tissues. As grasses grow and mature, the total yield of dry matter increases but they become fibrous with secondary cell wall deposition and lignification of sclerenchyma cells, resulting in low forage quality. Therefore, the improvement of cell wall digestibility is an important target in the breeding of forage grasses.

In *Arabidopsis thaliana*, 10 NAC transcription factors (TFs) belonging to the same phylogenetic subgroup—namely VASCULAR-RELATED NAC DOMAINs 1–7 (VNDs) and NAC SECONDARY WALL THICKENING PROMOTING FACTOR1 (NST1), NST2, and NST3 (the latter is also called SECONDARY WALL-ASSOCIATED NAC DOMAIN PROTEIN1)—have been identified as master transcriptional switches of secondary cell wall biosynthesis (Zhong and Ye 2014). These TFs, some of which are functionally redundant, activate a cascade of downstream TFs and genes, including those responsible for cellulose and lignin synthesis (Zhong and Ye 2014), in specific tissues such as xylem

vessels (Kubo et al. 2005), fibers (Mitsuda et al. 2007; Zhong et al. 2006, 2007) and anther endothecium (Mitsuda et al. 2005). When the function of these TFs is suppressed or disrupted, the differentiation of xylem vessels is inhibited (Kubo et al. 2005) or secondary cell wall formation is lost in those specific tissues (Mitsuda et al. 2005, 2007; Zhong et al. 2006, 2007).

Through the comparison of secondary cell wall formation networks in the dicot *Arabidopsis* and the monocot rice, TFs have been classified as common to both species or specific to one (Hirano et al. 2013). Several OsSWNs have been identified as orthologs of *Arabidopsis* VNDs and NSTs (Zhong et al. 2011). *OsSWN1* promoter activity is high in sclerenchyma tissues of culms and leaf veins and is lower in xylem vessels, whereas *OsSWN2* promoter activity shows the reverse (Chai et al. 2015; Yoshida et al. 2013). An *OsSWN2* chimeric repressor was generated to induce a loss-of-function phenotype of *OsSWN2* (Yoshida et al. 2013). In this chimeric repressor, the *OsSWN2* gene was fused with a modified ethylene-responsive element binding factor-associated amphiphilic repression (EAR)-like motif repression domain (*SRDX*) derived from the *SUPERMAN* gene of *Arabidopsis* (Hiratsu et al. 2003) to suppress the expression of target genes of *OsSWN2*. Transgenic rice expressing this chimeric repressor under the control of the *OsSWN1* promoter had reduced thickening of the secondary cell wall in leaf sclerenchyma cells, resulting in increased forage digestibility (Yoshida et al. 2013). The use of the *OsSWN1* promoter, which is highly active in sclerenchyma cells, would allow to avoid undesirable effects on plant growth expected from altering xylem cells. In this study, to improve forage digestibility, the *OsSWN2* chimeric repressor was introduced into tall fescue, secondary cell formation was measured and forage digestibility was evaluated.

2.2: Materials and methods

Vector construct

To produce the chimeric repressor, the coding region of the *OsSWN2* gene (*Os08g0115800*) without the stop codon was fused with the *SRDX* sequence (LDLDLELRLGFA) (Hiratsu et al. 2003) and placed under the control of the *OsSWN1* (*Os06g0131700*) promoter as described previously (Yoshida et al. 2013). This construct was transferred into the binary vector pBCKH carrying the hygromycin

phosphotransferase (*hpt*) gene as a selectable marker (Mitsuda et al. 2006) (Fig. 2.1). The binary vector was introduced into *Agrobacterium tumefaciens* strain EHA105 by electroporation with a Gene Pulser II (Bio-Rad, Hercules, CA, USA).

Medium components

Callus induction, culture and co-cultivation medium consisted of MS salts and vitamins (Murashige and Skoog 1962), 30 g L^{-1} sucrose, 1 g L^{-1} casein hydrolysate, 0.2 mg L^{-1} 6-benzylaminopurine and 2 g L^{-1} Gelrite (Wako, Osaka, Japan). In addition, the callus induction medium contained 5 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D; pH 5.8); the callus culture medium contained 2 mg L^{-1} 2,4-D (pH 5.8); and the co-cultivation medium contained 2 mg L^{-1} 2,4-D, 10 g L^{-1} glucose, and 100 μM acetosyringone (pH 5.2). Regeneration medium was MS medium containing 30 g L^{-1} sucrose, 0.2 mg L^{-1} kinetin and 4 g L^{-1} Gelrite (pH 5.8).

Plant transformation

Embryogenic calluses were induced from mature seeds of the forage cultivar ‘Nanryo’. Sterilized seeds were placed on the callus induction medium in the dark at 25°C and transferred to the fresh medium after 4 weeks. *A. tumefaciens* was grown in YEP medium (10 g L^{-1} peptone, 10 g L^{-1} yeast extract, 5 g L^{-1} NaCl, 2 mmol L^{-1} $MgCl_2$, pH 7.2) supplemented with antibiotics with agitation in the dark at 28°C for 20 h. The *Agrobacterium* suspension was collected by centrifugation (1500 ×*g*) and resuspended at an OD_{600} of 0.1 in AAM medium (Hiei et al. 1994). Calluses were treated with the *Agrobacterium* suspension for 5 min and divided into small pieces during this step. Excess suspension was removed by blotting the calluses on sterile filter paper, and the infected calluses were co-cultivated on sterile filter paper on the co-cultivation medium in the dark at 25°C for 4 days. The calluses were rinsed with 250 mg L^{-1} carbenicillin in sterile water and placed on the callus culture medium supplemented with 100 mg L^{-1} hygromycin and 250 mg L^{-1} carbenicillin in the dark at 25°C. The calluses were transferred to the fresh medium after 4 weeks. After 8 weeks in total, hygromycin-resistant calluses were placed on the regeneration medium 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. Regenerated plants were transferred to soil and grown in a greenhouse at 20–23°C. They were vernalized to induce flowering in a cold room under short-day conditions at 4°C for 8 weeks and then returned to the greenhouse.

Southern blot analysis

Genomic DNA was extracted from leaves by the cetyltrimethylammonium bromide (CTAB) method (Murray and Thompson 1980) with minor modifications. DNA was digested with *Hin*dIII (Fig. 2.1), separated in 0.8% agarose gels at 30 V, and blotted onto a positively charged nylon membrane (Roche Diagnostics, Mannheim, Germany). The *hpt* probe was amplified from the binary vector with a PCR digoxigenin (DIG) probe synthesis kit (Roche Diagnostics) and primers hpt-F (5′–CGAAGAATCTCGTGCTTTCA–3′) and hpt-R (5′–TCCATCACAGTTTGCCAGTG–3′). Southern blot analysis was performed as described in the DIG manual.

RT-PCR analysis

Total leaf RNA was extracted with an RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. It was treated with deoxyribonuclease (Wako, Osaka, Japan) according to the manufacturer's instructions. To check genomic DNA contamination of RNA, PCR amplification was carried out in a 14 μL mixture containing 250 ng of RNA treated with deoxyribonuclease, 1.4 μL of 10 × Ex Taq buffer, 0.7 μL of each 20 μM primer for actin, 1.1 μL of dNTP mixture (with each dNTP at 2.5 mM), and 0.25 U TaKaRa Ex Taq (TakaRa, Shiga, Japan). RT-PCR amplification was carried out in a 50 μL reaction mixture (the final volume) using illustra Ready-to-Go RT-PCR Beads (GE Healthcare UK, Buckinghamshire, England) according to the manufacturer's instructions. First-strand cDNA was synthesized from 500 ng total RNA with 500 ng first-strand primer, $pd(T)_{12\text{-}18}$, supplied in the kit at 42°C for 30 min, and the mixture was incubated at 95°C for 5 min to inactivate the reverse transcriptase. RT-PCR was performed with the following primers: 5′–CTTACTCCTCCAGCGACGAC–3′ and 5′–CAAACGGAGTTCTAGATCCA–3′ (SRDX-R) for *OsSWN2-SRDX*, and 5′–GATTCTGGTGATGGTGTCAG–3′ (actin-F) and 5′–

ATCCAGACACTGTACTTCCT–3′ (actin-R) for *actin* as an internal control. After incubation, 1.5 μL of each 20 μM primer was added to the mixture. PCR was then performed, using 32 cycles of 30 s at 95°C (denaturation), 30 s at 55°C (annealing), and 40 s at 72°C (extension) and a final extension for 7 min at 72°C. PCR products were separated by electrophoresis in 2% agarose gels.

Scanning electron microscopy

The base of the first leaf at the three-leaf stage and the upper part of the second internode below the apex at the floret opening stage were sampled. Tissues were held in a slit made in a piece of wood, sliced into 100-μm sections on a plant microtome (MTH-1; Nippon Medical & Chemical Instruments, Osaka, Japan) and coated with gold in an MSP-1S Magnetron sputter coater (Vacuum Device, Ibaraki, Japan). The sections were observed under a scanning electron microscope VE8800 (Keyence, Osaka, Japan) at an accelerating voltage of 5 kV. Thickness of secondary cell walls was measured 15 cells in each tissue.

Dry matter digestibility

Triplicate clones of transgenic plants with brittle internodes selected by phenotypic analyses and of wild-type plants were used. Plants were cut during the vegetative stage, and expanded leaves that regrew were harvested 5 weeks after routing. Stems, which may contain a small amount of leaf sheath on their surface, were collected by removing leaf blades from plants harvested at the floret opening stage. The samples were dried at 70°C for at least 2 days and ground into powder. Dry matter digestibility was analyzed by a modified enzymatic method as described previously (Abe 1988). In brief, approximately 0.5 g of the powder was boiled in 10 mL of distilled water for 1 min on a hot plate and cooled, and 35 mL of enzyme solution (0.26% cellulase and 0.013% α-amylase in 0.1 M acetate buffer, pH 5.8) was added. The mixture was then incubated at 40°C with shaking for 17.5 h and filtered through filter paper (Toyo 5A, 125 mm in diameter). The residue on the filter paper was washed three times with distilled water, dried at 105°C overnight, then cooled and weighed in a desiccator. Digestibility was calculated as:

$$100 - 100 \times (A' - A) / S \times (G / 100)$$

where A' = weight of the container + filter paper + residue, A = weight of the container + filter paper, S = weight of the sample and G = dry matter percentage.

Acid detergent fiber

Acid detergent fiber (ADF) was analyzed as described by Van Soest (1963). In brief, 50 mL of acid detergent solution (2% CTAB in 1N H_2SO_4) was added to approximately 0.5 g of the sample (the same that was used for measuring dry matter digestibility), and the mixture was placed on a heating unit for 60 min. The mixture was then filtered through a fritted glass crucible (1G–2; Sansyo, Tokyo, Japan). The residue on the crucible was washed seven times with distilled water and three times with acetone, and was dried at 105°C overnight. After cooling and weighting in a desiccator, the crucible with the residue was held at 550°C for 3 h in a muffle furnace. After cooling, the ash was weighed. ADF was calculated as:

$$100 \times (A - B) / S \times (G / 100)$$

where A = weight of the crucible + residue, B = weight of the crucible + ash, S = weight of the sample and G = dry matter percentage.

Determination of lignin content

The first leaves at the three-leaf stage and the second internodes at the floret opening stage were used. Alcohol-insoluble residue (AIR) was prepared as described by Sakamoto and Mitsuda (2015). In brief, samples were fixed in methanol and treated twice with methanol at 80°C for 10 min, twice with acetone at 70°C for 5 min, and twice with methanol/chloroform (1:1, *v*/*v*) at 70°C for 5 min. After rinsing with 99.5% ethanol three times, the samples were dried at 65°C overnight. Dried samples, which were considered as AIR, were pulverized into powder by using a stainless-steel bead (6 mm; Biomedical Science, Inc., Tokyo, Japan) and three zirconia beads (3 mm; Nikkato, Inc., Osaka, Japan) with a Shake Master NEO grinder (Biomedical Science, Inc.), the remaining starch in the powder was removed with amylase as described previously (Sakamoto and Mitsuda 2015). Destarched samples were subjected to

two-step sulfuric acid hydrolysis, and the residue (acid-insoluble lignin) was rinsed with ultrapure water and then weighed. Absorption of the supernatant at 210 nm was measured to calculate the concentration of acid-soluble lignin by using a lignin extinction coefficient of 110 L g^{-1} cm^{-1}.

Phloroglucinol staining

The base of the first leaf at the four-leaf stage and the upper part of the third internode from the apex of the stem at the late flowering stage were sampled. Tissues were sliced into 60-μm sections (leaves) or 50-μm sections (internodes) on MTH-1. The sections were stained with phloroglucinol as described by Zhang et al. (2006) with minor modifications: they were treated with fresh 5% phloroglucinol in 70% ethanol for 10 min, immersed in 50% HCl for 2 min, and observed under a microscope (BX50; Olympus, Tokyo, Japan).

2.3: Results

Production of transgenic tall fescue

A total of 27 transgenic plants were generated from calluses of three different genotypes (N1, nos. 1–4; N2, nos. 5–25; N3, nos. 26, 27). Since tall fescue is an outcrossing species, cultivars consist of various genotypes with different genetic background. Wild-type plants regenerated from non-transgenic calluses of the same genotypes were used as negative controls. The number of integrated gene copies, determined by Southern blot analysis with the *hpt* probe, ranged from one to at least nine (Fig. 2.2). The various hybridization patterns confirmed that the plants were derived from independent transformation events. Expression of *OsSWN2-SRDX* was detected in leaves from 24 of the 27 transgenic plants, but not in wild-type plants (Fig. 2.3). One *OsSWN2-SRDX* plant (no. 19) died during examination, so 23 transgenic plants expressing *OsSWN2-SRDX* were analyzed.

Phenotypic analyses

Stems of wild-type plants stood straight at flowering stage (Fig. 2.4a, c), whereas those of seven *OsSWN2-SRDX* plants (nos. 2, 3, 5, 7, 9, 21, 27) drooped gradually with maturation from elongation

stage to flowering stage (Fig. 2.4b, e). Six plants (nos. 4, 5, 8, 13, 18, 21) had semi-dwarf or dwarf phenotypes at flowering stage (Fig. 2.4d). Five plants (nos. 2, 4, 7, 9, 21) had fewer stems and two of them (nos. 2, 4) had shorter leaves than wild-type plants (Fig. 2.4d, e). Besides these phenotypes, the seven drooping plants, one dwarf plant (no. 4) and one semi-dwarf plant (no. 18) had brittle internodes that were more easily broken by bending than those of wild-type plants (Fig. 2.4f, g). No relationship between phenotypes and copy number was apparent (Fig. 2.2).

In comparison with wild-type plants, *OsSWN2-SRDX* plants with brittle internodes had significantly thinner secondary cell walls in sclerenchyma cells of the interfascicular fibers of internodes and in cortical fiber cells located between epidermal cells and vascular bundles of leaf veins (Fig. 2.5). In general, cell wall thickness is positively correlated with the AIR/fresh weight (FW) ratio if the cell wall is manipulated (Sakamoto and Mitsuda 2015; Sakamoto et al. 2016). The AIR/FW ratio was lower in stems of three plants (nos. 2–4; Fig. 2.6a) and in leaves of six plants (nos. 2–4, 5, 7, 21; Fig. 2.6b) than in those of wild-type plants; this difference may reflect thinner cell walls in sclerenchyma cells of *OsSWN2-SRDX* plants. In stems of three plants (nos. 5, 7, 21) generated from N2, AIR/FW was not reduced (Fig. 2.6a), suggesting that other phenotypic feature(s) such as cell wall thickness in the epidermis and/or the number of vascular bundles could be affected in this genotype.

Evaluation of forage digestibility

Dry matter digestibility of the brittle plants increased by a mean of 11.8% (range, 9.1%–15.8%) in stems of five plants (nos. 3, 5, 7, 21, 27; Fig. 2.7a) and by 6.8% (3.2%–11.2%) in leaves of six plants (nos. 2–5, 7, 21; Fig. 2.7b) compared with the mean of each source plant.

ADF represents indigestible fiber, which consists mainly of cellulose and lignin (Colburn and Evans 1967). ADF is frequently used to estimate energy value because it is highly correlated with cell wall digestibility. ADF was reduced by 25.2% (17.7%–29.1%) in stems of five plants (nos. 3, 5, 7, 21, 27; Fig. 2.7c) and by 21.6% (14.2%–27.0%) in leaves of three plants (nos. 2–4; Fig. 2.7d) relative to the mean of each source plant. Although ADF in leaves of the other three plants (nos. 5, 7, 21) was not reduced (Fig. 2.7d), their digestibility increased significantly (Fig. 2.7b).

Analysis of lignin accumulation

In stems, the content of acid-soluble lignin was significantly increased in three plants (nos. 5, 7, 21) and acid-insoluble lignin was reduced by 57.3% (56.2%–58.8%) in three plants (nos. 2, 7, 21) relative to the mean of each source plant (Fig. 2.8a). Consequently, the total lignin content was reduced by 48.0% (46.4%–49.3%) in three plants (nos. 2, 7, 21; Fig. 2.8a). In leaves, acid-soluble lignin was significantly increased in two plants (nos. 5, 21), but acid-insoluble lignin was not reduced (Fig. 2.8b).

Cross-sections of mature internodes and leaves were stained with phloroglucinol, a reagent traditionally used to detect cinnamyl aldehyde residues in lignin (Wardrop 1971). Metaxylem vessels and bundle sheath fibers were deeply stained in both wild-type and *OsSWN2-SRDX* plants (Fig. 2.9). Interfascicular fibers of internodes in wild-type plants were deeply stained (Fig. 2.9a), as were those in three plants (nos. 2–4; data not shown), but those in the other three plants (nos. 5, 7, 21) were lightly stained (Fig. 2.9b). Cortical fiber cells in leaf veins were less densely stained in *OsSWN2-SRDX* than in wild-type plants (Fig. 2.9c, d). Reduced staining may result from lignin with fewer cinnamyl aldehyde end groups. These obvious differences in staining indicate that lignin accumulation was reduced in sclerenchyma cells of the *OsSWN2-SRDX* plants.

2.4: Discussion

Of 23 transgenic plants expressing *OsSWN2-SRDX*, nine plants had brittle internodes (Fig. 2.4g); the stems of seven of the nine plants drooped gradually with maturation because of low mechanical strength (Fig. 2.4b, e). This phenotype resembled bending characteristics of the *brittle culm* (*bc*) mutants caused by the reduction in cellulose content and cell wall thickness in rice (Li et al. 2003), barley (Kokubo et al. 1991) and maize (Ching et al. 2006; Sindhu et al. 2007). Stem lignin contents in the mutants of rice *bc1* and maize *brittle stalk2*, which have loss-of-function phenotypes of COBRA-like glycosylphosphatidylinositol-anchored protein genes, were higher than that in the wild-type plants (Li et al. 2003; Sindhu et al. 2007). In contrast, those in some barley *bc* mutants and the *OsSWN2-SRD*X plants were similar to or lower than in wild-type plants (Kokubo et al. 1991; Fig. 2.8a). Thus, the brittle phenotype is caused by the reduced deposition of cellulose in cell walls, whereas lignin content is not

directly related to brittleness. The undesirable phenotypes were the reduction in the number or length (or both) of stems and/or leaves in some *OsSWN2-SRDX* plants (Fig. 2.4d, e). These phenotypes might be caused by the inhibition of water transport, because the *OsSWN1* promoter could have some activity in xylem vessels (Yoshida et al. 2013).

Stems have lower forage quality than leaves (Collins 1988). As grasses mature, the proportion of leaves declines, thus reducing forage quality (Stone et al. 1960). Therefore, the increase in stem digestibility is desirable to limit the loss of forage quality at the reproductive stage. The dry matter digestibility of the *OsSWN2-SRDX* plants with brittle internodes was increased by 11.8% in stems (Fig. 2.7a) and by 6.8% in leaves (Fig. 2.7b) compared with wild-type plants. Averaged data across species including bermudagrass [*Cynodon dactylon* (L.) Pers.], hybrid ryegrass (*Lolium hybridum* Hausskn.), Italian ryegrass, orchardgrass, pubescent wheatgrass [*Thinopyrum intermedium* subsp. *barbulatum* (Schur) Barkw. & D.R. Dewey], smooth bromegrass (*Bromus inermis* Leyss.) and switchgrass (*Panicum virgatum* L.) indicate that a 1% increase in digestibility results in a 3.2% increase in daily weight gain of beef cattle (Casler and Vogel 1999). Therefore, the digestibility of the *OsSWN2-SRDX* plants was greatly improved. ADF was reduced by 25.2% in stems (Fig. 2.7c) and by 21.6% in leaves (Fig. 2.7d) relative to the wild-type. These results indicate that the reduction in the content of indigestible cellulose and lignin, which are components of secondary cell walls in sclerenchyma cells, increased forage digestibility (Fig. 2.7). Stems are more fibrous than leaves because of the predominance of sclerenchyma cells such as those in interfascicular fibers. Therefore, the improvement was greater in stems than in leaves (Fig. 2.7).

Dry matter digestibility of the leaves of three plants (nos. 5, 7, 21) was significantly increased (Fig. 2.7b) but the ADF was not reduced (Fig. 2.7d). The content of acid-insoluble lignin was unchanged but that of acid-soluble lignin was increased (Fig. 2.8b). Therefore, the changes in acid-soluble lignin might result in increased digestibility. Although the impact of lignin composition on digestibility is still unsolved, lignin composition that altered by genetic manipulation showed a better relationship with forage digestibility in some species (Li et al. 2008). On the other hand, lignin content and composition remained unchanged in stems of one plant (no. 3) and in leaves of two plants (nos. 3, 4) whose dry

matter digestibility increased (Figs. 2.7a, b, 2.8a, b). Because the ADF was significantly reduced (Fig. 2.7c, d), the digestibility may have been improved by the reduction of indigestible cellulose fiber content. These phenotypic variations might be due to partial repression by the chimeric repressor in the complex pathways of secondary cell wall biosynthesis.

In tall fescue, there has been two reports of transgenic plants with down-regulated CAD and COMT using each antisense gene driven by maize ubiquitin promoter; their digestibility increased significantly (Chen et al. 2003, 2004). These plants had decreased lignin content and altered lignin composition from reducing activity of individual enzymes in lignin biosynthesis pathway. No significant change in level of cellulose was observed in the *CAD* down-regulated plants (Chen et al. 2003). In this study, the author demonstrated a new strategy to improve forage digestibility by altering the expression of SWN TFs and genes for secondary cell wall biosynthesis. The *OsSWN2* chimeric repressor coordinately inhibits the expression of genes for the secondary cell wall biosynthesis in sclerenchyma cells. Therefore, reduction of secondary cell wall thickness can decrease indigestible lignin and/or cellulose in the *OsSWN2-SRDX* plants, resulting in increased forage digestibility.

The presence of OsSWN homologs in various plants (Zhong and Ye 2014) suggests that transcriptional networks regulated by SWNs are conserved among vascular plants. Hence, the strategy to improve forage digestibility by reducing secondary cell wall formation could also be applicable to other forage grasses. On the other hand, dry matter yield in some transgenic lines might be reduced due to the changes in cell wall components. Forage quality is weakly negatively correlated with yield (Casler 1986). However, a previous study demonstrated that a 2.8% increase in digestibility led to a 23% increase in dry matter intake and an 11% increase in milk production in continuous grazing of a tall fescue cultivar with high palatability even though dry matter yield was 8% lower than the control (Emile et al. 1992). Therefore, the *OsSWN2-SRDX* plants could potentially increase livestock performance. Next, the *OsSWN2-SRDX* plants should be further examined to evaluate their agronomic traits, including forage digestibility, dry matter yield, disease resistance, environmental stress tolerance and seed production, under field conditions.

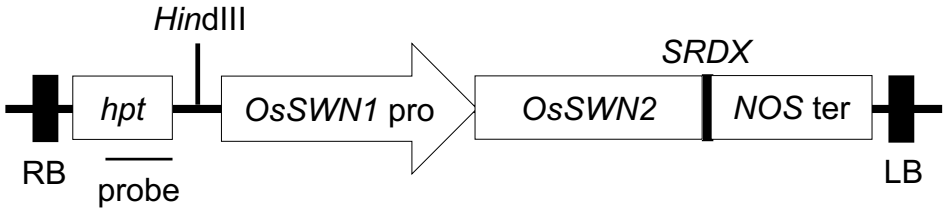

Figure 2.1 Schematic representation of the chimeric repressor of *OsSWN2*. RB, right border; *hpt*, hygromycin phosphotransferase gene; *OsSWN1* pro, promoter of the *OsSWN1* gene; *OsSWN2*, coding region of the *OsSWN2* gene without the stop codon; *SRDX*, modified EAR-like motif repression domain; *NOS* ter, terminator of the nopaline synthase gene; LB, left border. The probe for Southern blot analysis is indicated by a horizontal line.

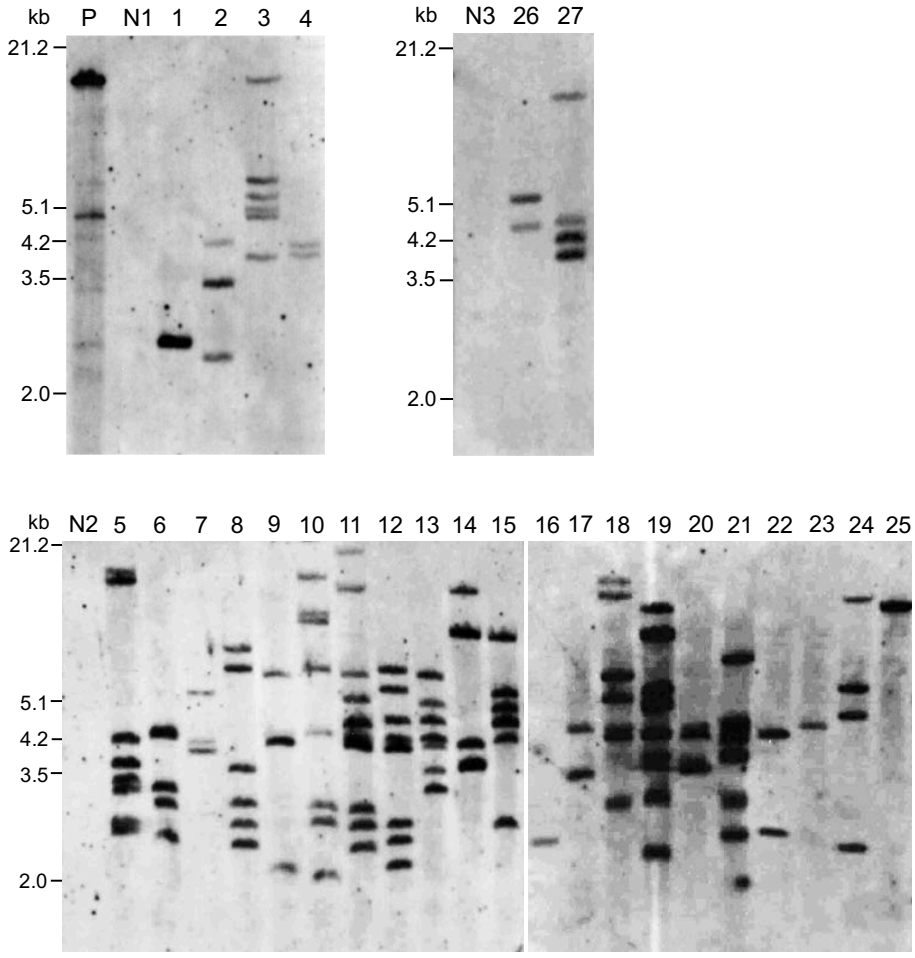

Figure 2.2 Southern blot analysis with the *hpt* probe. P, binary vector; N1–N3, plants regenerated from non-transgenic calluses of three different genotypes; 1–4, transgenic plants generated from N1 callus; 5–25, transgenic plants generated from N2 callus; 26 and 27, transgenic plants generated from N3 callus.

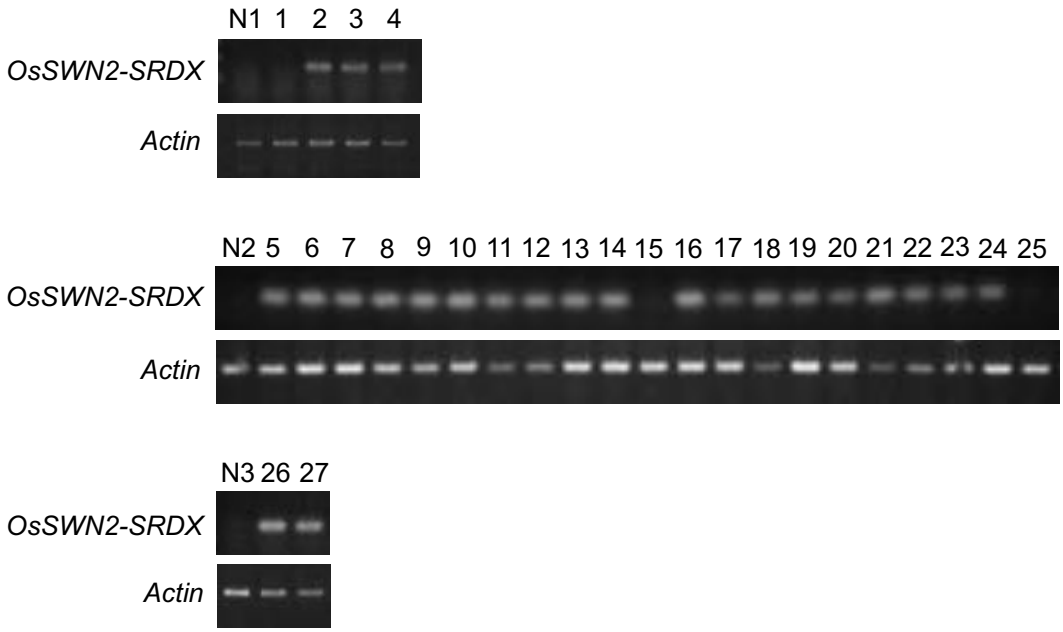

Figure 2.3 Expression of *OsSWN2-SRDX* in leaves analyzed by RT-PCR. N1–N3, plants regenerated from non-transgenic calluses; 1–4, transgenic plants generated from N1 callus; 5–25, transgenic plants generated from N2 callus; 26 and 27, transgenic plants generated from N3 callus. Actin transcript was used as an internal control.

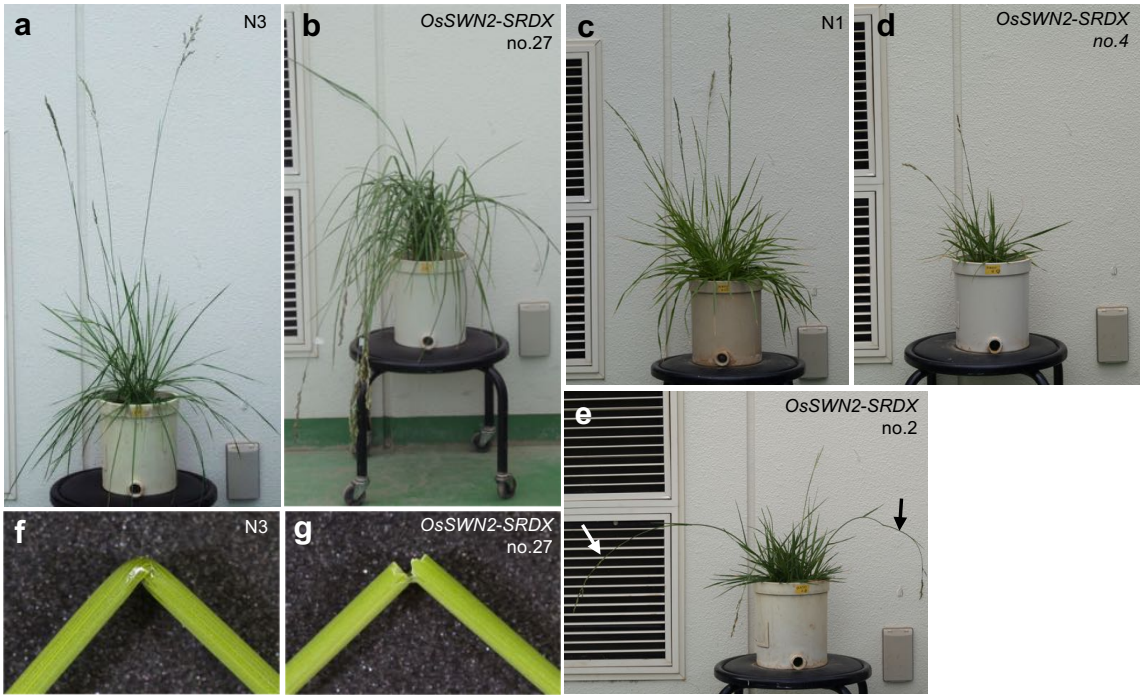

Figure 2.4 Phenotypes of (a, c, f) wild-type and (b, d, e, g) transgenic plants. (a–e) Plants at flowering stage. (f, g) Internodes. Arrows indicate drooping stems.

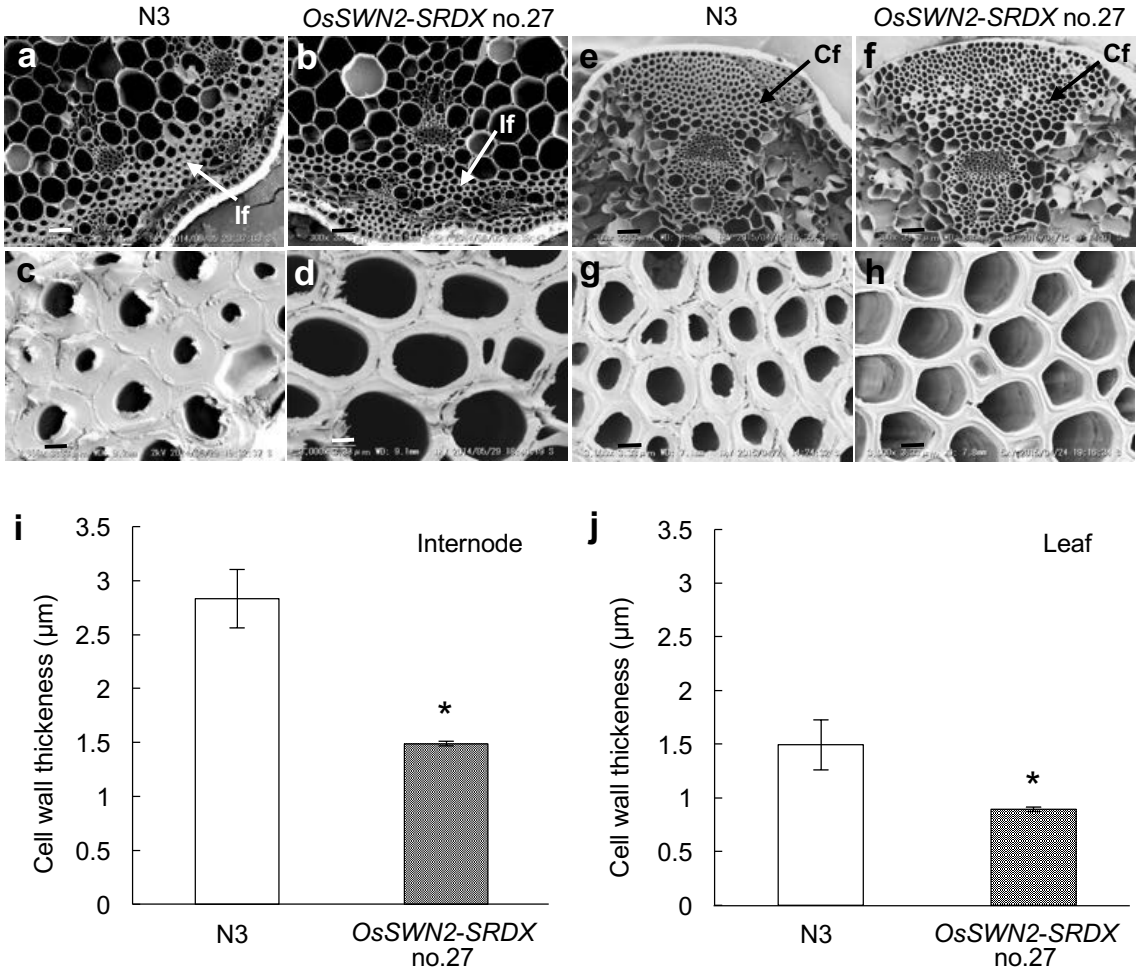

Figure 2.5 Secondary cell walls in cross-sections of (a, c, e, g) wild-type and (b, d, f, h) transgenic plants examined by scanning electron microscopy. (a, b) Internodes. If, Interfascicular fiber. (c, d) Interfascicular fibers of the internodes. (e, f) Leaf veins. Cf, Cortical fiber cells. (g, h) Cortical fiber cells between epidermal cells and the vascular bundle of the leaf vein. Bars indicate 33.3 μm (a, b, e, f) or 3.33 μm (c, d, g, h). (i) Cell wall thickness in internodes. (j) Cell wall thickness in leaves. Error bars represent SD of triplicate experiments. * Significantly different from the source plant by Welch's *t*-test at $P < 0.05$.

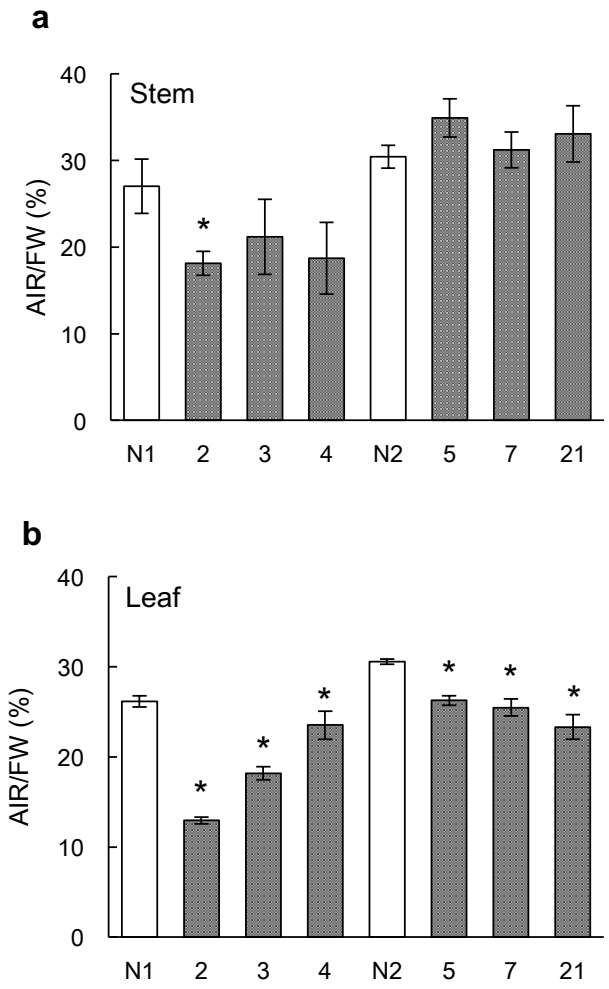

Figure 2.6 The AIR/FW ratio in (a) stems and (b) leaves. N1, N2, plants regenerated from non-transgenic calluses; 2–4, *OsSWN2-SRDX* plants generated from N1 callus; 5, 7, 21, *OsSWN2-SRDX* plants generated from N2 callus. Error bars represent SD of quadruplicate experiments. * Significantly different from the respective source plant by Dunnett's test at $P < 0.05$.

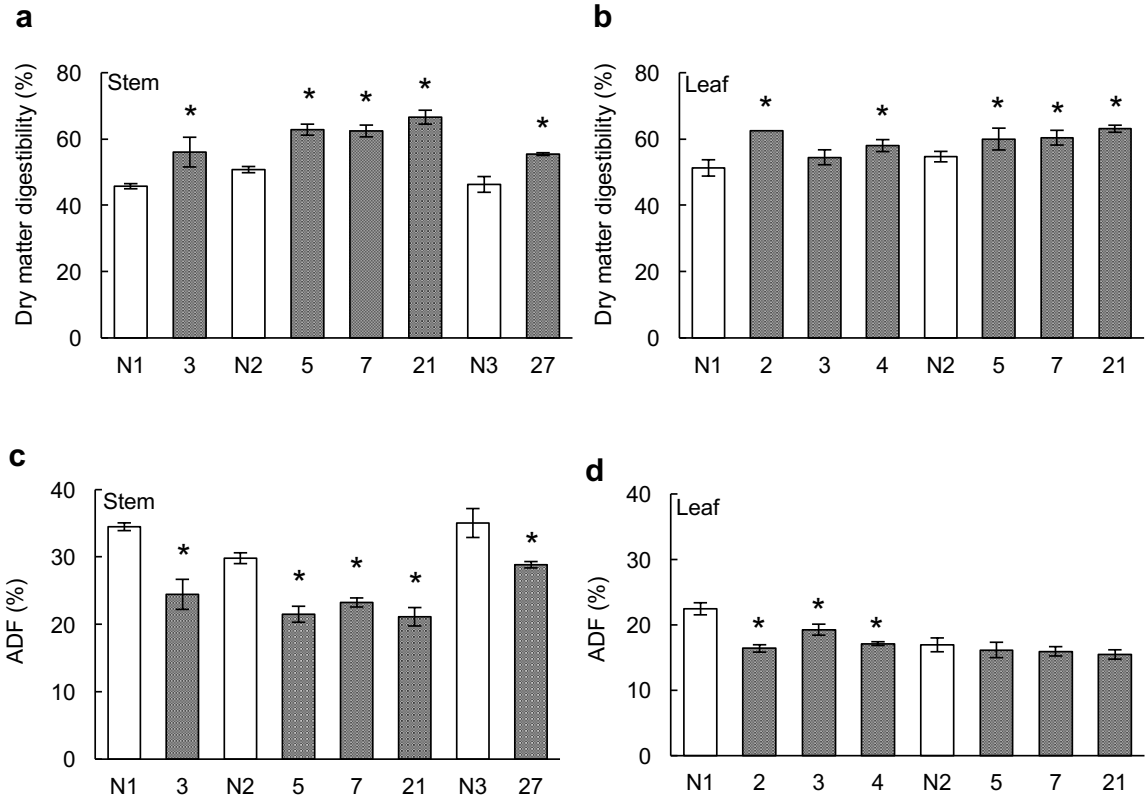

Figure 2.7 Digestibility of wild-type and transgenic plants. (a) Dry matter digestibility of stems. (b) Dry matter digestibility of leaves. (c) ADF of stems. (d) ADF of leaves. N1–N3, plants regenerated from non-transgenic calluses; 2–4, *OsSWN2-SRDX* plants generated from N1 callus; 5, 7, 21, *OsSWN2-SRDX* plants generated from N2 callus; 27, *OsSWN2-SRDX* plant generated from N3 callus. Error bars represent SD of triplicate clones. No error bar is displayed for no. 2 in b because of duplicate clones. * Significantly different from the respective source plant by Dunnett's test or Student's *t*-test at $P < 0.05$.

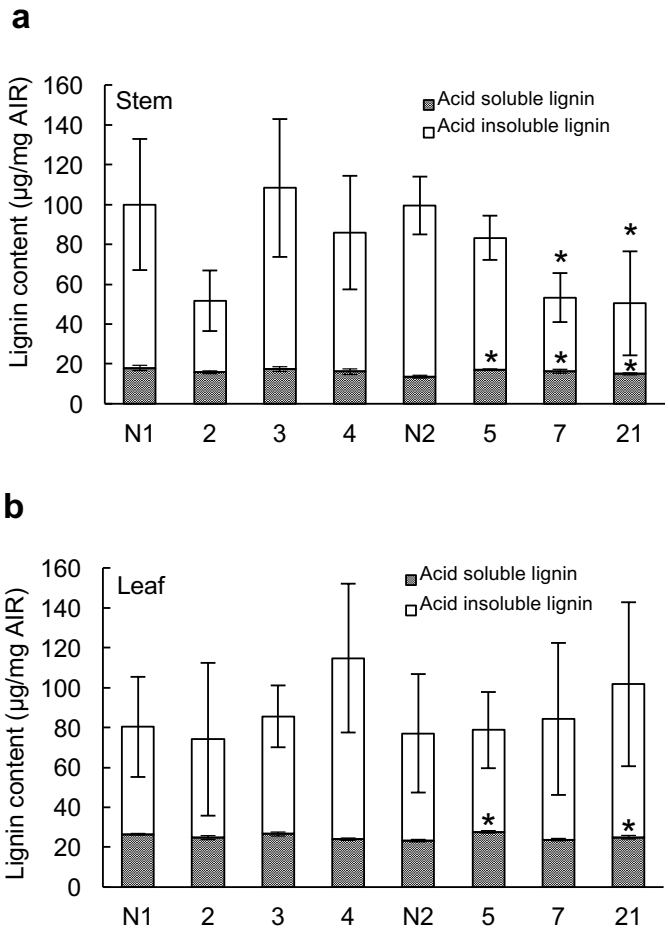

Figure 2.8 Lignin content in the AIR of (a) stems and (b) leaves. N1, N2, plants regenerated from non-transgenic calluses; 2–4, *OsSWN2-SRDX* plants generated from N1 callus; 5, 7, 21, *OsSWN2-SRDX* plants generated from N2 callus. Error bars represent SD of quadruplicate experiments. * Significantly different from the respective source plant by Dunnett's test at $P < 0.05$.

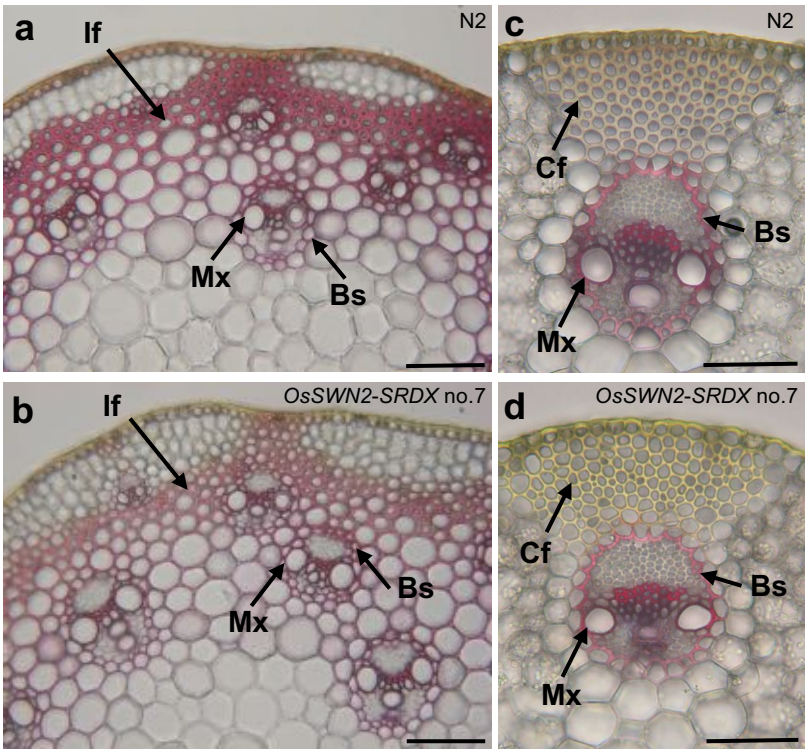

Figure 2.9 Phloroglucinol staining (pink) in cross-sections of (a, c) wild-type and (b, d) transgenic plants. (a, b) Internodes. If, Interfascicular fiber; Mx, metaxylem vessel, Bs, bundle sheath fiber. Bars indicate 100 μm. (c, d) Leaf veins. Cf, Cortical fiber cells between epidermal cells and the vascular bundle. Bars indicate 50 μm.

Chapter 3: Production of herbicide-resistant tall fescue by introducing a mutated *Oryza sativa* acetolactate synthase gene

3.1: Introduction

Weeds reduce plant yield and quality because they compete with cultivated plants for water, nutrients, sunlight and space. Herbicide resistance allows easier weed elimination because otherwise it is difficult to control weeds without damaging desirable plants. Herbicide resistance can also provide economic and environmental benefits by reducing production costs, herbicide spraying and fuel use (Green 2012). In 2016, Herbicide-resistant (HR) plants comprised 88% of all GM crops (ISAAA 2016). The use of HR turfgrass is promising on golf courses, athletic fields and lawns. Transgenic creeping bentgrass (Fei and Nelson 2004) and Kentucky bluegrass (Blume et al. 2010) resistant to glyphosate are already deregulated in the USA or their deregulation is pending.

ALS is the first enzyme in the biosynthetic pathway leading to the branched-chain amino acids (leucine, isoleucine and valine). It is the target of at least five structurally distinct classes of herbicides, namely PCs, sulfonylureas, imidazolinones, triazolopyrimidine sulfonamides and sulfonylamino-carbonyl-triazolinones (Shimizu et al. 2002). Plants sprayed with these herbicides cannot synthesize the essential amino acids and therefore die. Resistance to ALS-inhibiting herbicides has in most cases been conferred by either single or double amino acid substitutions in ALS (Kawai et al. 2007). ALS genes with spontaneous mutations have been isolated from plants (Kawai et al. 2007). Different types of mutations confer resistance to different classes of ALS-inhibiting herbicides (Kawai et al. 2007). Some mutations that confer resistance to imidazolinones have been used commercially in bread wheat, maize, oilseed rape, rice and sunflower (*Helianthus annuus* L.) (Tan et al. 2005).

Mutated *ALS* genes have also been used as plant-derived selectable markers to avoid the use of bacterial antibiotic-resistant genes. Selectable markers are critical for the selection of transgenic cells and elimination of non-transgenic cells after genetic transformation. Although selectable marker genes are unnecessary after the selection of transgenic cells, HR genes can be used both to select transgenic cells and confer herbicide resistance in plants regenerated from these cells. Mutated *ALS* genes derived

from *Arabidopsis* have been used as selectable markers in tobacco (*Nicotiana tabacum* L.) (Gabard et al. 1989), rice (Li et al. 1992), potato (*Solanum tuberosum* L.) (Andersson et al. 2003), oilseed mustard [*Brassica juncea* (L.) Czern.] (Ray et al. 2004) and maize (Zhang et al. 2005). Mutated rice *ALS* genes have been used in rice (Okuzaki et al. 2007; Osakabe et al. 2005; Taniguchi et al. 2010), bread wheat (Ogawa et al. 2008) and soybean (Tougou et al. 2009). Because some amino acid substitutions that confer herbicide resistance are well conserved in plant ALSs (Kawai et al. 2007), they work in various plant species. An *ALS* gene with two point mutations (W548L and S627I) has been isolated from a rice callus; these mutations confer strong resistance to bispyribac-sodium (BS), a PC herbicide (Kawai et al. 2007). The S627I mutation had not been previously reported. The single S627I mutation confers weak resistance to BS but strong resistance to two other PCs, PM and pyrithiobac-sodium (PS) (Kawai et al. 2008).

In tall fescue transformation, bacterial *hpt* is the most frequently used selectable marker (Wang and Ge 2006); no plant-derived gene has yet been used. Here, transgenic tall fescue was selected by using the *O. sativa ALS* gene containing the S627I single mutation [*OsALS* (sm)] under the control of the *OsALS* promoter and terminator, and its herbicide resistance was evaluated. The aim of this study was to assess the effectiveness of the mutated *ALS* gene in conferring herbicide resistance in tall fescue.

3.2: Materials and methods

Dose-response tests in tall fescue calluses

Embryogenic calluses of three different genotypes were induced from the shoot tips of the turf cultivar 'Tomahawk' germinated *in vitro* in the dark at 25°C. An approximately 0.25 g callus (wet weight) was placed on the callus culture medium, which was the same as that used in Chapter 2 except that contained no casein hydrolysate but contained PM or PS (Kumiai Chemical Industry Co., Tokyo, Japan) at 0, 0.01, 0.1, 1 and 10 μM. The calluses were transferred to the fresh medium after 4 weeks. After 8 weeks in total, the callus weight was measured.

Vector construct

The binary vector R-2 carried the *OsALS* (sm) gene under the control of the *OsALS* promoter and terminator (Kumiai Chemical Industry Co. 2010) (Fig. 3.1). Accession number of the nucleotide sequence that includes the *OsALS* gene, promoter and terminator is AP005841. The binary vector was introduced into *A. tumefaciens* strain EHA105 by electroporation with the Gene Pulser II (Bio-Rad).

Plant transformation

Tall fescue was transformed as described in Chapter 2.2 with minor modifications. Calluses were divided into small pieces, placed on callus culture medium for 3 days, and treated with the *Agrobacterium* suspension for 1 min. Infected calluses were selected on callus culture medium containing 0.25 μM PM in the dark at 25°C. Casein hydrolysate was excluded because it supplies the essential amino acids not biosynthesized under PM treatment. The calluses were transferred to the fresh medium after 4 weeks. After 8 to 10 weeks in total, PM-resistant calluses were placed on the regeneration medium without PM because regeneration tended to be inhibited by incubation with PM. The transformation efficiency was calculated as: the number of transgenic plants carrying the *OsALS* (sm) gene/the number of calluses infected with *Agrobacterium* × 100. The spikelets of one of the transgenic plants were crossed with those of a CMS line 'B30' within a paper bag. The T_1 seeds were harvested from the CMS plant.

Southern blot analysis

Southern blot analysis was performed as described in Chapter 2.2. Genomic DNA was digested with *Bam*HI (Fig. 3.1). The *OsALS* probe was amplified from the binary vector with primers 5′–AGGTGTCACAGTTGTTGA–3′ and 5′–GAGGGGATTTAAAGTCTTGG–3′ to cover the *OsALS* terminator (Fig. 3.1).

Herbicide application

Leaves were sprayed with 50 μM PM solution to run off in the greenhouse. After 2 weeks, spraying was

repeated. The response to the herbicide was observed 45 days after treatment.

Measurement of acetolactate synthase activity

ALS activity was analyzed by colorimetric enzymatic assay (Osakabe et al. 2005) with minor modifications. In brief, leaves (50 mg) were cut into small pieces and incubated in 4 mL of pretreatment solution [25% MS basal medium (Murashige and Skoog 1962), 500 µM 1,1-cyclopropanedicarboxylic acid and 10 mM pyruvic acid sodium salt] with or without 0.1 µM PM under fluorescent light at 30°C for 24 h. Leaf pieces were then transferred to a new tube and frozen for 1 h. Subsequently, 220 µL of 0.025% Triton X-100 solution was added, and the tube was heated at 60°C for 10 min. After incubation, 200 µL of the supernatant was mixed with 20 µL of 5% H_2SO_4 and incubated at 60°C for 30 min. Then, 100 µL of 5% 1-naphthol dissolved in 2.5N NaOH and 100 µL of 0.5% creatine were added, and the mixture was incubated at 37°C for 30 min. The color of the reaction mixture was observed, and the absorbance at 530 nm was measured by a spectrophotometer.

3.3: Results

Production of transgenic tall fescue by pyriminobac selection

Tall fescue calluses responded differently to PM or PS (Fig. 3.2a). The growth of calluses on 1 µM PM was completely inhibited whereas that on 1 µM PS was inhibited only partially (Fig. 3.2a). Because of the high PM sensitivity of tall fescue calluses, PM was considered suitable for the selection of transgenic calluses carrying the *OsALS* (sm) gene. PM-resistant calluses were clearly distinguishable after 8 to 10 weeks in the presence of PM (Fig. 3.2b).

Southern blot analysis showed that one or more *OsALS* (sm) gene copies were present in all regenerated plants (Fig. 3.3). Although a hybridization band was observed in the wild-type plant N1, this band was not detected in the transgenic plants nos. 1–4 generated from N1 (Fig. 3.3). Therefore, it was concluded that the band was an artifact such as antibody for the detection of DIG-labeled compounds. The absence of other bands was detected in either of the two wild-type plants (N1, N2; Fig. 3.3) indicated that the probe was specific to the *OsALS* construct. The transformation efficiency of three

different genotypes was 3.9% (1.8%–6.5%).

Herbicide resistance of transgenic tall fescue

Wild-type and *OsALS* (sm) plants were sprayed on their leaves with PM solution (Fig. 3.4a, b). Wild-type plants stopped growing immediately after spraying, but the leaves remained green because PM is not a quick-acting herbicide and does not immediately kill tall fescue. At 45 days after herbicide treatment, leaf growth did not recover and some parts of each plant died (Fig. 3.4c). On the other hand, all *OsALS* (sm) plants were unaffected by the treatment and continued to grow regardless of PM treatment (Fig. 3.4d).

Colorimetric assay estimates ALS activity in plant tissues by assessing acetoin accumulation (Gerwick et al. 1993). Red or pink indicates high accumulation of acetoin produced by ALS activity, and yellow or brown indicates low accumulation. When leaf tissues were incubated without PM, samples from both wild-type and *OsALS* (sm) plants were deep pink (Fig. 3.5a) and their ALS activity was almost equivalent (Fig. 3.5b). In the assay with PM, samples from wild-type plants were yellow, indicating that their ALS activity was inhibited by PM, whereas samples from *OsALS* (sm) plants were light pink (Fig. 3.5a). ALS activity in *OsALS* (sm) plants was lower with PM than without PM (Fig. 3.5b), probably because PM treatment inhibited endogenous ALS. In the presence of PM, all *OsALS* (sm) plants showed higher ALS activity than did wild-type plants (Fig. 3.5b). ALS activity in the *OsALS* (sm) plants with PM was approximately 60% (44%–74%) of that of wild-type plants without PM, whereas that of wild-type plants with PM was approximately 32%. These results indicate that the *OsALS* (sm) plants produced the OsALS (sm) protein, which was resistance to PM.

Molecular and phenotypic analyses of T_1 progeny

Transgenic plant no. 1, which had six copies of the *OsALS* (sm) gene (Fig. 3.3), was crossed with a wild-type plant. Southern blot analysis confirmed the presence of the *OsALS* (sm) gene in 26 of 27 T_1 plants but not in the wild-type plant (Fig. 3.6a). The T_1 plants had various copy numbers, which did not follow a Mendelian pattern (Fig. 3.6a). The T_1 plant no. 1 with one copy of the transgene was obtained;

five (bands 2–6) of the six bands showed a 1:1 segregation ratio (Fig. 3.6a). These results suggest that the transgenes were integrated into separate unlinked loci.

Of the 27 T_1 plants, 26 were resistant to PM, whereas one plant (no. 23) died after PM treatment. The *OsALS* (sm) gene was present in all resistant plants but not in the susceptible plant (Fig. 3.6a). ALS activity in the leaf tissues of PM-resistant plants incubated with PM was lower than that in leaf tissues from wild-type plant without PM (Fig. 3.6b). However, it was twice that of the wild-type plant with PM (Fig. 3.6b). These results indicate that PM-resistant plants produced OsALS (sm) protein. Thus, the molecular and phenotypic analyses confirmed the inheritance and stable expression of the *OsALS* (sm) gene in the T_1 progeny.

3.4: Discussion

Transgenic tall fescue with strong resistance to BS in greenhouse tests was produced previously by using a rice *ALS* gene with two point mutations under the control of the cauliflower mosaic virus 35S promoter (data not shown). On the other hand, other transgenic plants with multiple integrated transgenes was susceptible to the herbicide (data not shown). Strong expression driven by constitutive promoters tends to cause deleterious effects such as gene silencing in the presence of multiple copies of the transgene (Okuzaki et al. 2007). Here, transgenic tall fescue was produced using the *OsALS* (sm) gene under the control of the native *OsALS* promoter. ALS activity in transgenic and T_1 plants treated with PM was lower than that in wild-type plants in the absence of PM (Figs. 3.5b, 3.6b), likely because the *OsALS* promoter is not strong and is active in specific tissues (Osakabe et al. 2005). Half of the transgenic plants had multiple transgene copies (Fig. 3.3) but these plants survived after herbicide treatment (Fig. 3.4). Therefore, the native *ALS* promoter is suitable for stable expression of the transgene.

A cultivation system that depends on the application of a single herbicide would tend to increase the frequency of emergence of HR weeds through random mutations. In particular, there are more weed species that are resistant to ALS-inhibiting herbicides than to any other herbicides because the resistance is conferred by point mutations (Tranel and Wright 2002). The effective tactics to inhibit the generation of HR weeds are rotation and combination of several herbicides with different modes of action (Green

and Owen 2011). Since glyphosate, glufosinate and ALS-inhibiting herbicides belong to different families of chemicals (Tan et al. 2006), the use of a combination of several herbicides with different mechanisms is useful for delaying the generation of HR weeds. Next, it is necessary to produce stacked cultivars resistant to those herbicides.

Mutated *ALS* genes can be used as plant-derived selectable markers. In grass transformation, the most widely used selectable marker genes are those for antibiotic resistance, such as those for hygromycin phosphotransferase and neomycin phosphotransferase (Giri and Praveena 2015). These two genes pose no problems for humans (Lu et al. 2007; Nap et al. 1992). However, the Food Safety Commission of Japan (FSCJ) recommends considering alternative transformation methods that do not result in any residual antibiotic-resistant genes in food, where such techniques are available and demonstrated to be safe (FSCJ 2004). Since tall fescue calluses are highly sensitive to PM, transgenic calluses containing the *OsALS* (sm) gene were effectively selected by incubation with PM (Fig. 3.2). This study demonstrates that the *OsALS* (sm) gene can be used both for the production of HR tall fescue and as a selectable marker in the transformation.

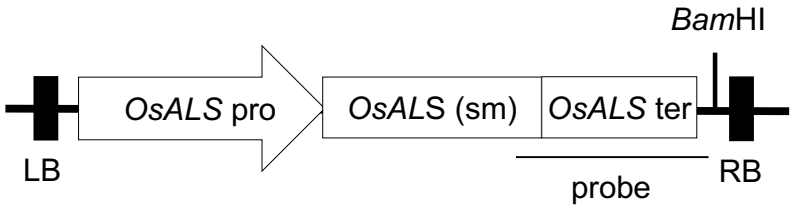

Figure 3.1 Schematic representation of the *OsALS* (sm) construct. LB, left border; *OsALS* pro, promoter of the *OsALS* gene; *OsALS* (sm), coding region of the *OsALS* gene containing the S627I mutation; *OsALS* ter, terminator of the *OsALS* gene; RB, right border. The probe for Southern blot analysis is indicated by a horizontal line.

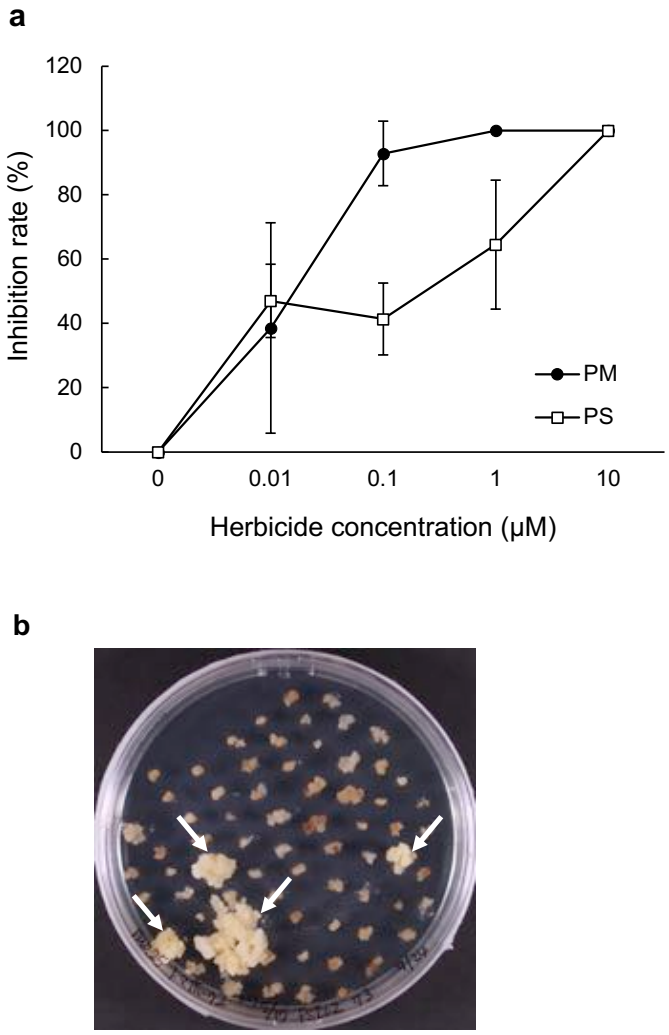

Figure 3.2 Production of transgenic tall fescue by PM selection. (a) Dose-response tests with wild-type calluses. Inhibition rate (%) = (1 – callus weight at each concentration (0–10 μM)/callus weight at 0 μM) × 100. Error bars represent SD of three genotypes. (b) Transgenic calluses selected by incubation on 0.25 μM PM for 10 weeks. Arrows indicate PM-resistant calluses.

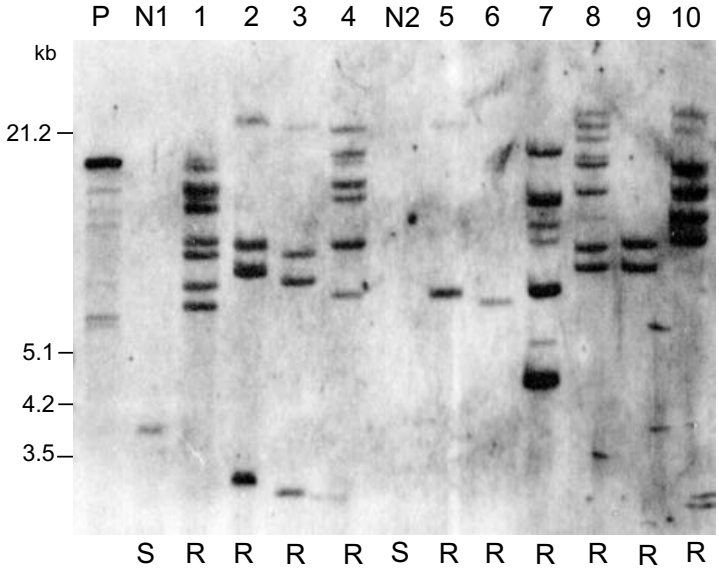

Figure 3.3 Southern blot analysis with the *OsALS* probe. P, binary vector; N1, N2, wild-type plants; 1–10, transgenic plants; R, resistant; S susceptible to PM.

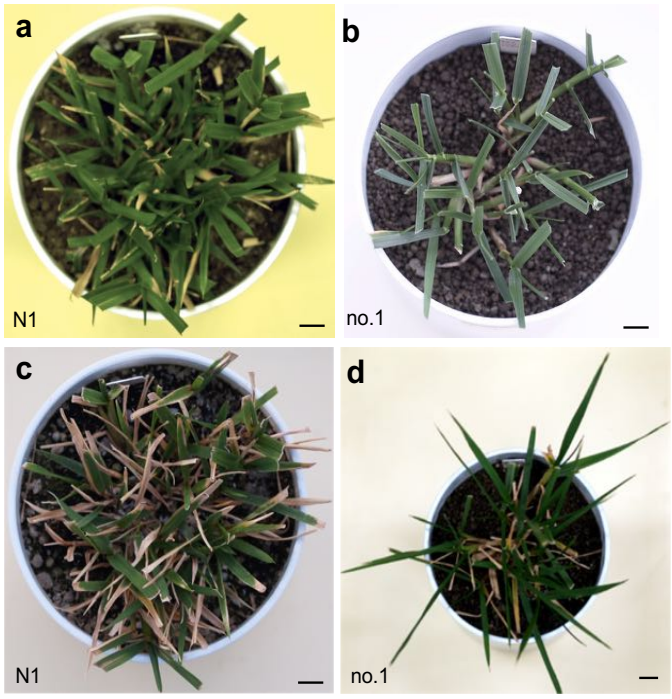

Figure 3.4 Herbicide resistance of (a, c) wild-type and (b, d) transgenic plants. (a, b) Plants before herbicide treatment. (c, d) Plants 45 days after herbicide treatment. Bars indicate 1 cm.

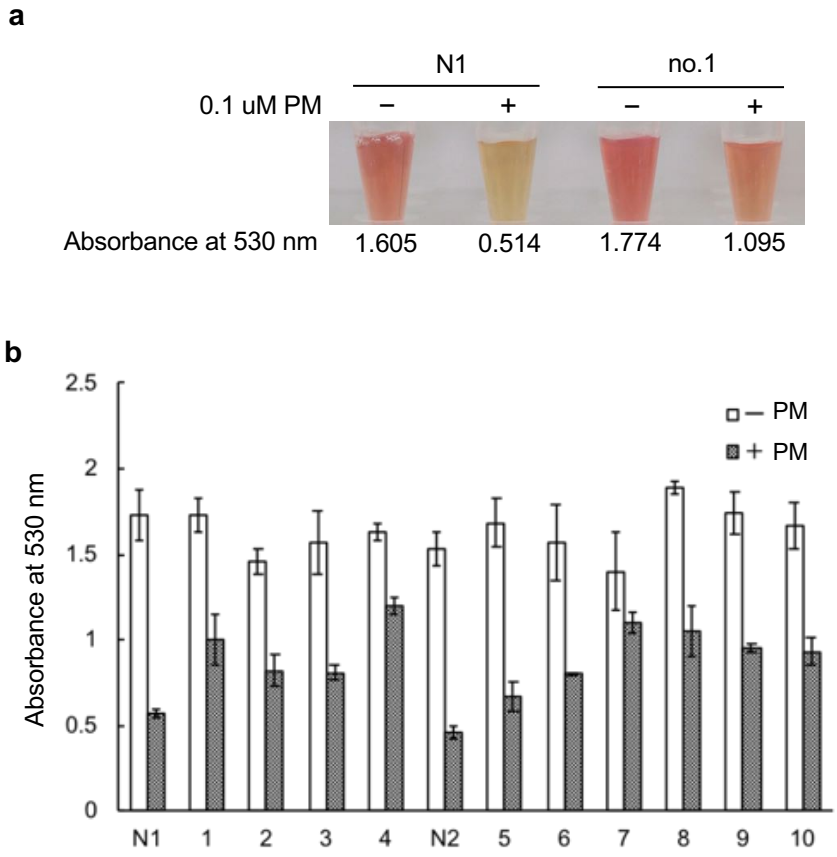

Figure 3.5 Colormetric enzymatic assay of leaves. (a) Acetoin accumulation. (b) ALS activity. N1, N2, wild-type plants; 1–10, transgenic plants. Error bars represent SE of triplicate experiments.

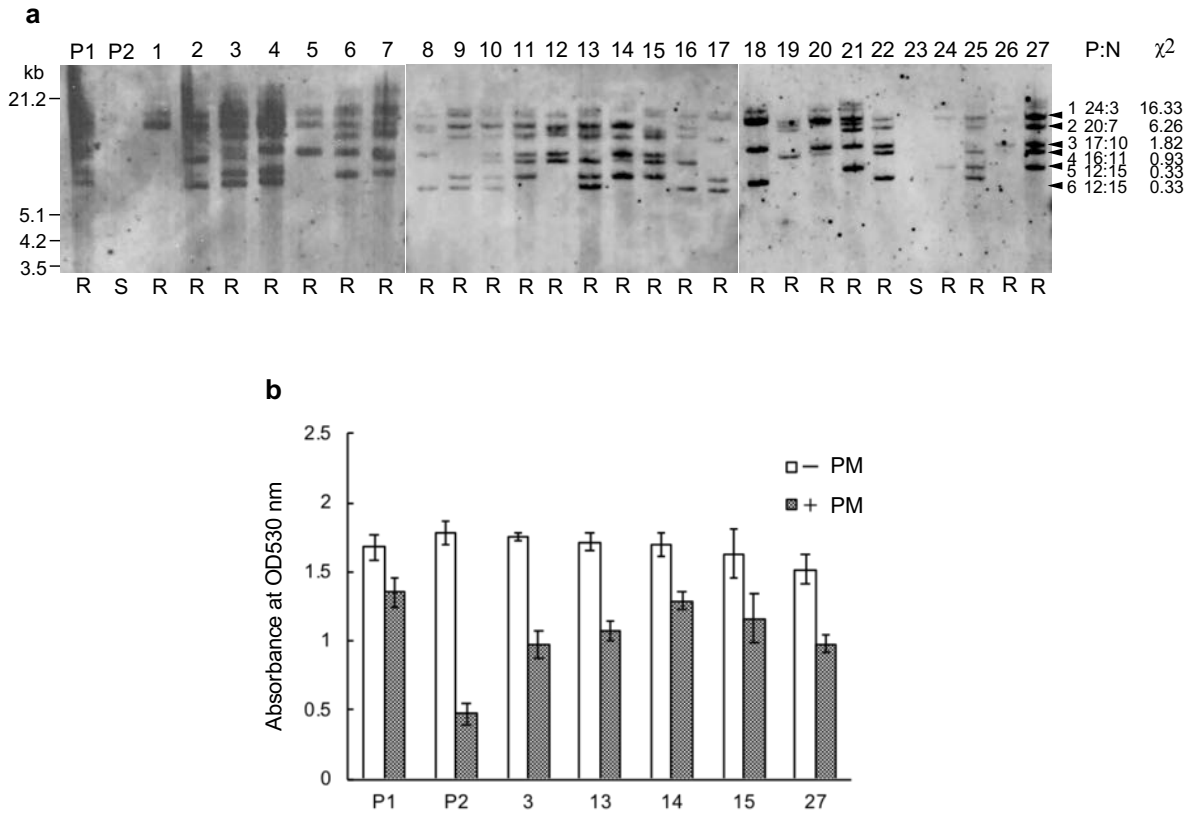

Figure 3.6 Inheritance of the *OsALS* (sm) gene in the T_1 progeny. (a) Southern blot analysis with the *OsALS* probe. P1, transgenic plant no. 1 (pollen parent); P2, wild-type plant (seed parent); 1–27, T_1 plants; R, resistant; S susceptible to PM. The segregation ratio of positive (P) to negative (N) plants was analyzed for each band by the chi-squared test. χ^2 (0.05) = 3.84; χ^2 (0.01) = 6.64. (b) ALS activity. Error bars represent SE of triplicate experiments.

Chapter 4: Induction of male sterility by chimeric repressors of *SUPERWOMAN1* and *OsMADS58* to prevent pollen flow

4.1: Introduction

Most forage and turf grasses are cross-pollinated and anemophilous because of their self-incompatibility. They disperse large amounts of pollen into the environment, and people susceptible to the allergenic proteins of grass pollen suffer from hay fever. One of the risks in the cultivation of transgenic grasses is the possibility that transgenes will be spread by crossing with sexually compatible wild species. A landscape-level study of transgenic creeping bentgrass carrying 5-enol-pyruvylshikimate-3-phosphate synthase gene as a marker found that most of the gene flow occurred within 2 km in the direction of prevailing winds (Watrud et al. 2004). The maximal gene flow distances observed were 21 km for sentinel plants and 14 km for resident plants (Watrud et al. 2004). This report played a role in tightening the regulatory process of the APHIS on outcrossing grasses; the procedures and requirements are stringent if transgenic grasses are allowed to flower in the field (Wang and Brummer 2012). Major cool-season grasses (including orchardgrass, perennial ryegrass, tall fescue and timothy) have been recently listed as industrial controlled alien species by the Japanese Ministry of the Environment (MOE) because they have the potential to become invasive in natural habitats and to affect the diversity of native species (MOE 2016). Therefore, grass pollen flow is an important issue not only for human health but also in terms of its environmental and ecological impacts.

Male sterility can prevent pollen flow. CMS has been found in wild plant populations; it occurs as a result of crosses in which the nuclear genome of one species is moved into the cytoplasmic background of another species (Hanson and Bentolila 2004). Although CMS is very useful, it is available only for a limited number of species because successive backcrossing, which is very labor intensive, is necessary to carry out interspecific exchange of cytoplasmic and nuclear genomes.

The “ABC model” has been proposed to explain how genes control identity of four floral organs (sepals, petals, stamens and carpels) in the dicots *A. thaliana* and *Antirrhinum majus* (Bowman et al. 1991; Coen and Meyerowitz 1991; Theiβen 2001). Most of the ABC genes encode MADS-box TFs.

Mutations of the ABC genes cause homeotic transformation of floral organs and therefore make it possible to improve floral traits. A combination of B- and C- class genes specifies the formation of stamens. *APETALA3* (*AP3*) and *PISTILLATA*, B-class genes of *Arabidopsis*, regulate stamen and petal identity (Goto and Meyerowitz 1994; Jack et al. 1992). In the *ap3* mutant, stamens and petals are homeotically transformed into carpels and sepals, respectively (Jack et al. 1992). *AGAMOUS* (*AG*), a C-class gene of *Arabidopsis*, plays an important role in organ identity of stamens and carpels, repression of A-class genes and floral meristem determinacy (Bowman et al. 1991; Lenhard et al. 2001; Lohmann et al. 2001; Yanofsky et al. 1990). In the *ag* mutant, stamens are homeotically transformed into petals, and central carpels are replaced by another agamous flower (Bowman et al. 1991; Yanofsky et al. 1990). Therefore, the loss-of-function phenotype of each *AP3* and *AG* results in male-sterile flowers.

Although the flower structure in monocots differs from that of dicots, the ABC model could be also applicable to monocots (Ma and dePamphilis 2000; Whipple et al. 2004). Maize *Silky1* gene (Ambrose et al. 2000) and rice *SPW1* gene (Nagasawa et al. 2003), orthologs of *AP3*, specify the identity of stamens and lodicules; the latter organs correspond to the petals of *Arabidopsis*. The functions of the *AG* gene have diversified and become partially redundant as a result of gene duplication in monocots. In maize, *ZAG1* regulates floral meristems and *ZMM2* determines organ identity (Mena et al. 1996). In rice, *OsMADS3* and *OsMADS58* genes have similar functions (Yamaguchi et al. 2006). Stamens in *silky1*, *spw1* and *osmads3* mutants and *OsMADS58* RNAi plants are homeotically transformed into other floral organs or changed morphologically because of suppression of these TFs, in each case resulting in male sterility (Ambrose et al. 2000; Nagasawa et al. 2003; Yamaguchi et al. 2006). RNAi technology was also applied to suppress *SPW1* in rice, but the frequency of the loss-of-function phenotype was low (Xiao et al. 2003). Transgenic plants expressing each chimeric repressor of *AP3* and *AG* in *Arabidopsis*, and *SPW1* in rice often have male-sterile phenotypes similar to those of the mutants of these TFs (Mitsuda et al. 2006).

If chimeric repressors can induce male sterility in tall fescue, they will be useful for preventing transgenic pollen dispersal. When an agronomically useful gene and a chimeric repressor in the same construct are transferred, they can simultaneously improve the target trait and induce male sterility in

transgenic plants. Of the three chimeric repressors of rice *AG* orthologs, *Os12g0207000*, *Os01g0886200* and *OsMADS58*, that of *OsMADS58* was found to be most suitable for inducing male sterility in rice (data not shown). In this study, to create male-sterile tall fescue plants, the chimeric repressors of *SPW1* and *OsMADS58* were introduced and the alteration of floret traits was examined.

4.2: Materials and methods

Phylogenetic analysis

The phylogenetic relationship of *AP3*- and *AG*-related *Arabidopsis* and monocot genes was analyzed with the ClustalW program (Thompson et al. 1994). Bootstrap values were calculated from 1000 replicates. Accession numbers of each nucleotide or amino acid sequence are 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).

Vector constructs

The coding regions of the *OsMADS58* and *SPW1* genes, without the stop codons, were each fused with the *SRDX* sequence (Hiratsu et al. 2003) and placed under the control of the rice *actin1* promoter (McElroy et al. 1990). The constructs for these chimeric repressors, *OsMADS58-SRDX* and *SPW1-SRDX*, were transferred into the binary vector pBCKH (Mitsuda et al. 2006) (Fig. 4.1). The binary vectors were introduced into *A. tumefaciens* strain EHA105 by electroporation with the Gene Pulser II (Bio-Rad).

Plant transformation

Genetic transformation of 'Tomahawk' was performed as described in Chapter 3.2 with minor modifications: transgenic calluses were selected on callus culture medium containing 100 mg L^{-1}

hygromycin for 8 weeks and placed on the regeneration medium with 100 mg L^{-1} hygromycin. Pollen grains were stained with 1% carmine in 45% acetic acid and observed under a light microscope.

Southern blot analysis

Southern blot analysis was performed as described in Chapter 2.2. Genomic DNA was digested with *Hin*dIII (Fig. 4.1). The *hpt* probe was amplified as in Chapter 2.2.

RT-PCR analysis

RT-PCR was performed as described in Chapter 2.2 with minor modifications. Total RNA was extracted from florets when spikelets had fully emerged. RT-PCR was performed with the following primers: 5′–AAGGCCTAGGAAAGATTAGA–3′ and SRDX-R (the same as in Chapter 2.2) for *OsMADS58-SRDX*, 5′–CGTACGAGACTCTGCAGCAGGA–3′ and SRDX-R for *SPW1-SRDX*, and actin-F and actin-R for *actin* (see Chapter 2.2). After first-strand cDNA synthesis, 2.5 μL of each 20 μM primer was added to the mixture. PCR was then performed, using 32 cycles of 30 s at 95°C (denaturation), 30 s at 55°C (annealing), 45 s at 72°C (extension) and a final extension for 7 min at 72°C.

4.3: Results

Production of transgenic tall fescue

An ortholog of *OsMADS58* in meadow fescue (GO793260, approximately 71% identity at the amino acid level) and that of *SPW1* in tall fescue (DT690242, approximately 74% identity) were found (Fig. 4.2), suggesting the existence of functional orthologs in *Festuca*.

A total of seven *OsMADS58-SRDX* and four *SPW1-SRDX* plants were obtained. No obvious abnormalities were found in the transgenic plants during the vegetative stage. The copy number of the integrated gene ranged from two to at least seven in *OsMADS58-SRDX* plants (Fig. 4.3a). In *SPW1-SRDX* plants, one to four transgene copies were present (Fig. 4.3b). Expression of *OsMAD58-SRDX* was detected in florets from all the *OsMADS58-SRDX* plants, but not in wild-type plants (Fig. 4.4a; data for A6 is not shown). Because the expression of *SPW1-SRDX* was detected in two of the four plants, only

these two plants (S1, S3; Fig. 4.4b) were analyzed.

Observation of floral organs in tall fescue expressing *SPW1-SRDX*

Stamens elongate out of the glumes upon floret opening and remain outside the glumes in wild-type florets (Fig. 4.5a). In *SPW1-SRDX* plants, the florets showed a cleistogamous (closed) phenotype in which anthers were not observed outside the glumes (Fig. 4.5b). Within the palea and lemma, wild-type florets have three stamens, one carpel with two stigmas and two lodicules at the lemma side of the floret (Fig. 4.5d). While the apparent structure of stamens and carpel were normal in the *SPW1-SRDX* plants (Fig. 4.5e), the lodicules were thin and abnormally elongated (Fig. 4.5h). Lodicules play an important role in floret opening by swelling to push open the lemma (Yoshida 2012) (Fig. 4.5g). Therefore, the cleistogamous phenotype was likely associated with the morphological change of the lodicules.

When pollen was mature in wild-type plants (Fig. 4.5m), anthers were dehiscent (Fig. 4.5j) and pollen was dispersed into the environment. Although no changes in the apparent structure of stamens were observed, the pollen was not produced in *SPW1-SRDX* plants (Fig. 4.5n). Thus, the anthers were not dehiscent (Fig. 4.5k) and the plants were male-sterile.

Observation of floral organs in tall fescue expressing *OsMADS58-SRDX*

In five of the seven *OsMADS58-SRDX* plants (A1–A3, A6, A7), the florets did not open and the anthers were not observed outside the glumes (Fig. 4.5c). In the cleistogamous phenotype of the *OsMADS58-SRDX* plants, the lodicules were transformed homeotically into lemma-like organs (Fig. 4.5i) and these abnormal lodicules were considerably larger than those of the wild-type and *SPW1-SRDX* plants (Fig. 4.5g–i). In contrast, the stamens and stigmas were smaller than those of the wild-type and *SPW1-SRDX* plants (Fig. 4.5d–f). Although each *SPW1-SRDX* and *OsMADS58-SRDX* plant showed a cleistogamous phenotype associated with morphological changes to the lodicules (Fig. 4.5b, c), the phenotype of *OsMADS58-SRDX* plants differed considerably from that of *SPW1-SRDX* plants (Fig. 4.5h, i). The pollen was either not produced or was immature in the *OsMADS58-SRDX* plants (Fig. 4.5o); thus, the anthers were not dehiscent (Fig. 4.5l). Hence, the cleistogamous phenotype seen in the *OsMADS58-*

SRDX plants was associated with male sterility.

A mild phenotype characterized only by male sterility was found in two of the seven *OsMADS58-SRDX* plants (A4, A5; Fig. 4.6f, h) compared with wild-type plants (Fig. 4.6e, g). The florets of these two plants opened because the lodicules were morphologically normal, and the stamens and carpel were also normal (Fig. 4.6b, d) and similar to those in wild-type plants (Fig. 4.6a, c).

4.4: Discussion

SPW1 plays an important role in controlling stamen specification because stamens are homeotically transformed into carpels in the rice *spw1* mutant (Nagasawa et al. 2003) and *SPW1-SRDX* rice (Mitsuda et al. 2006). The phenotype of the maize *silky1* mutant is similar to that of the rice *spw1* mutant (Ambrose et al. 2000), indicating that the function of B-class genes is well conserved in monocots. In rice, a wide range of phenotypes have been observed in *SPW1* RNAi plants (Xiao et al. 2003). Approximately 44% of flowers in these plants are similar to those of different rice *spw1* mutant (Xiao et al. 2003), which carries a missense mutation (I45T) in *SPW1* (Yoshida et al. 2007). In the *spw1* mutant, the stamens are not affected, but the lodicules are homeotically transformed into elongated lodicule-glume mosaic organs, thereby producing cleistogamous flowers (Yoshida et al. 2007). The phenotype of *SPW1-SRDX* tall fescue was also similar to that of the rice *spw1* mutant with a I45T mutation (Fig. 4.5e, h), suggesting that a gene identified in rice is useful for producing a similar phenotype in tall fescue.

OsMADS58 may play a role in controlling stamen specification because homeotic transformation of stamens into lodicules was observed in rice expressing an RNAi construct against *OsMADS58* (Yamaguchi et al. 2006) or *OsMADS58-SRDX* (data not shown). Intriguingly, *OsMADS58-SRDX* tall fescue did not exhibit the reiterated lodicule-like structure, which was observed in *OsMADS58-SRDX* rice (data not shown). This difference might be due to the functional divergence of C-class genes between rice and tall fescue or to only a partial suppression by the chimeric repressor. *AG* is crucial for several aspects of floral development in *Arabidopsis* (Bowman et al. 1991; Lenhard et al. 2001; Lohmann et al. 2001; Yanofsky et al. 1990). These functions are split between *OsMADS3* and *OsMADS58* in rice (Yamaguchi et al. 2006). In maize, the functions of *AG* may also be separated

between two genes, *ZAG1* and *ZMM2* (Mena et al. 1996). These studies suggest that the functions of C-class genes have diversified in monocots and become partially redundant following gene duplication, implying that monocot C-class genes may play yet unknown roles in floral development. The cleistogamous phenotype, which was unexpectedly observed in *OsMADS58-SRDX* tall fescue (Fig. 4.5c), might be due to the suppression of another C-class gene whose expression pattern and roles have not yet been identified. It remains to be seen whether C-class genes are present in tall fescue, although an ortholog of an *AG*-related gene from the *Festuca* species was found (Fig. 4.2). Otherwise, the ubiquitous promoter used in this study might have induced this unexpected phenomenon.

Cleistogamous phenotypes associated with morphological changes to the lodicules were observed in both *SPW1-SRDX* and *OsMADS58-SRDX* tall fescue (Fig. 4.5b, c). They would be useful to prevent pollen flow in self-pollinating plants such as rice (Yoshida et al. 2007). However, cleistogamous phenotypes could not be used in outcrossing grasses because their florets require opening for crossing and seed production. On the other hand, the mild phenotype of *OsMADS58-SRD*X plants that exhibited only male sterility (Fig. 4.6) might be suitable as breeding materials for preventing pollen flow. For this purpose, it is necessary to confirm the stable inheritance of *OsMADS58-SRD*X in the progeny. This study demonstrated that novel male-sterile phenotypes were produced due to the alteration of floret traits by using chimeric repressors in tall fescue.

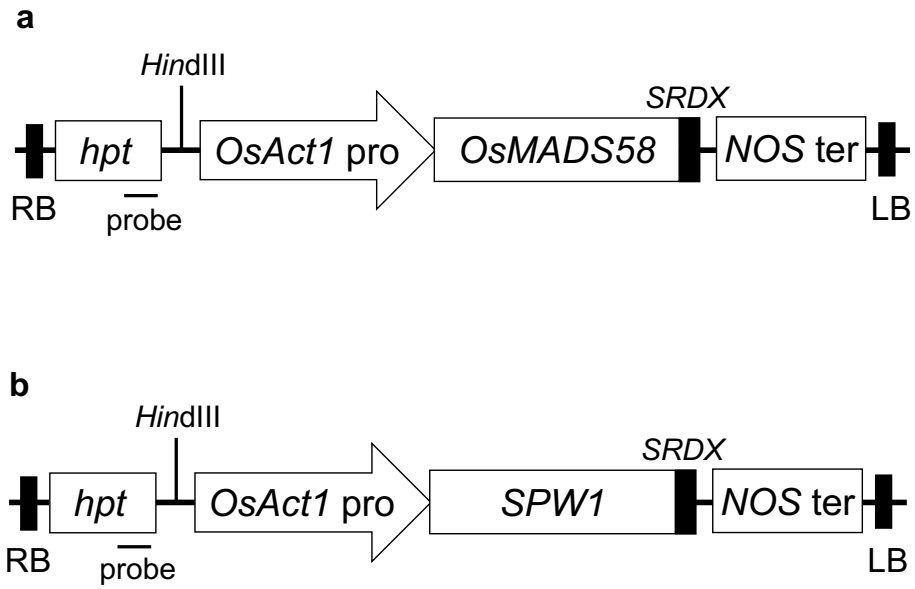

Figure 4.1 Schematic representation of the chimeric repressors of (a) *OsMADS58* and (b) *SPW1*. RB, right border; *hpt*, hygromycin phosphotransferase gene; *OsAct1* pro, promoter of the rice *actin1* gene; *OsMADS58*, coding region of the *OsMADS58* gene without the stop codon; *SRDX*, modified EAR-like motif repression domain; *NOS* ter, terminator of the nopaline synthase gene; LB, left border; *SPW1*, coding region of the *SUPERWOMAN1* gene without the stop codon. Probes for Southern blot analysis are indicated by horizontal lines.

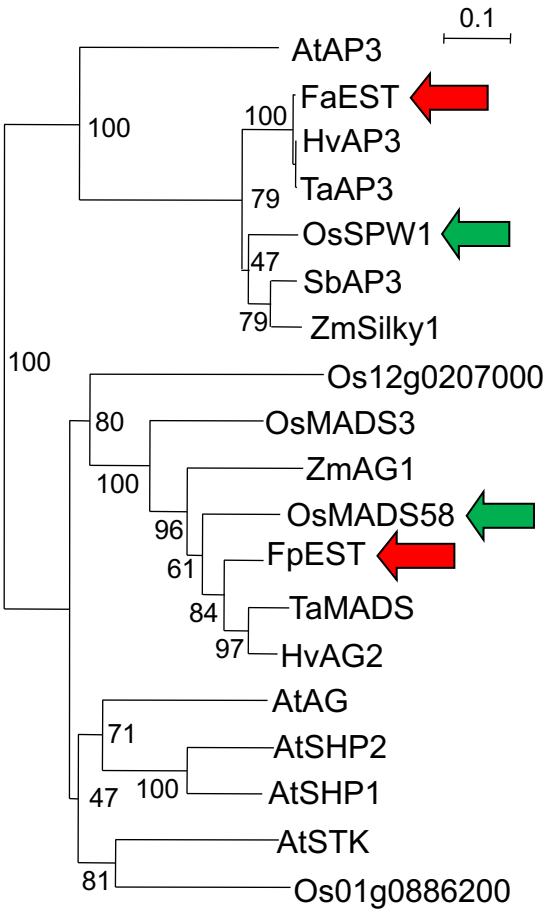

Figure 4.2 Neighbor-joining phylogenetic tree of *AP3*- and *AG*-related *Arabidopsis* and monocot genes. Numbers at the nodes indicate bootstrap percentage of 1000 replicates. At, *Arabidopsis*; Fa, tall fescue; Fp, meadow fescue; Hv, barley; Os, rice; Sb, sorghum; Ta, bread wheat; Zm, maize; EST, expressed sequence tag. Red arrows indicate partial EST sequences isolated from floral organs of *Festuca* species, which are orthologs of rice genes (green arrows). Scale bar indicates the number of substitutions per site.

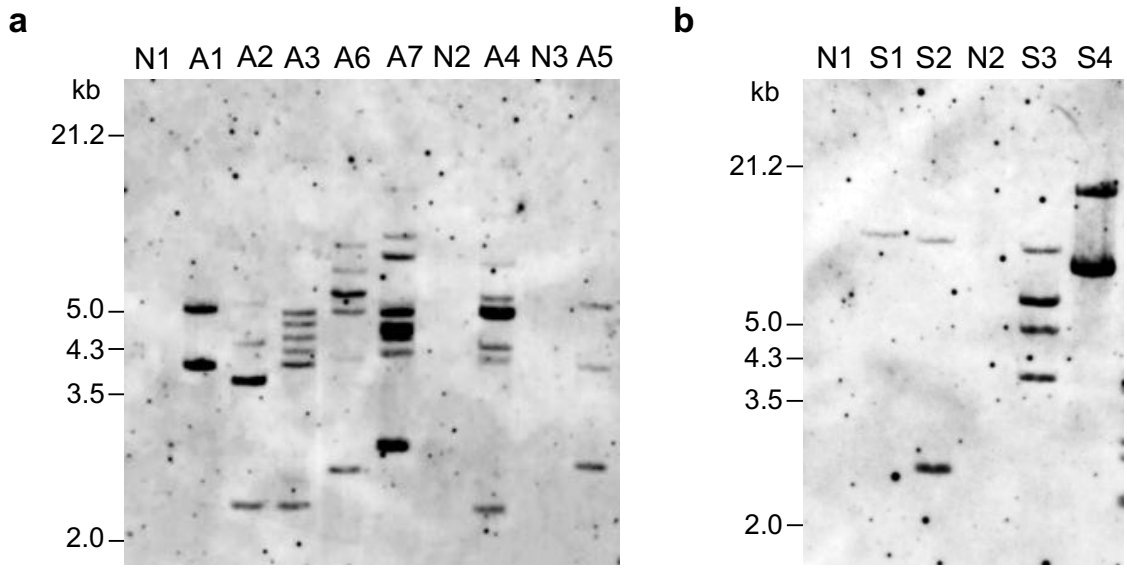

Figure 4.3 Southern blot analysis of tall fescue transformed with (a) *OsMADS58-SRDX* or (b) *SPW1-SRDX*. N1–N3, plants regenerated from non-transgenic calluses; A1–A7, *OsMADS58-SRDX* plants; S1–S4, *SPW1-SRDX* plants. Genomic DNA was hybridized with the *hpt* probe.

Figure 4.4 Expression of (a) *OsMADS58-SRDX* and (b) *SPW1-SRDX* in florets analyzed by RT-PCR. N1, N2, plants regenerated from non-transgenic calluses; A1–A5 and A7, *OsMADS58-SRDX* plants; S1–S4, *SPW1-SRDX* plants. Actin transcript was used as an internal control.

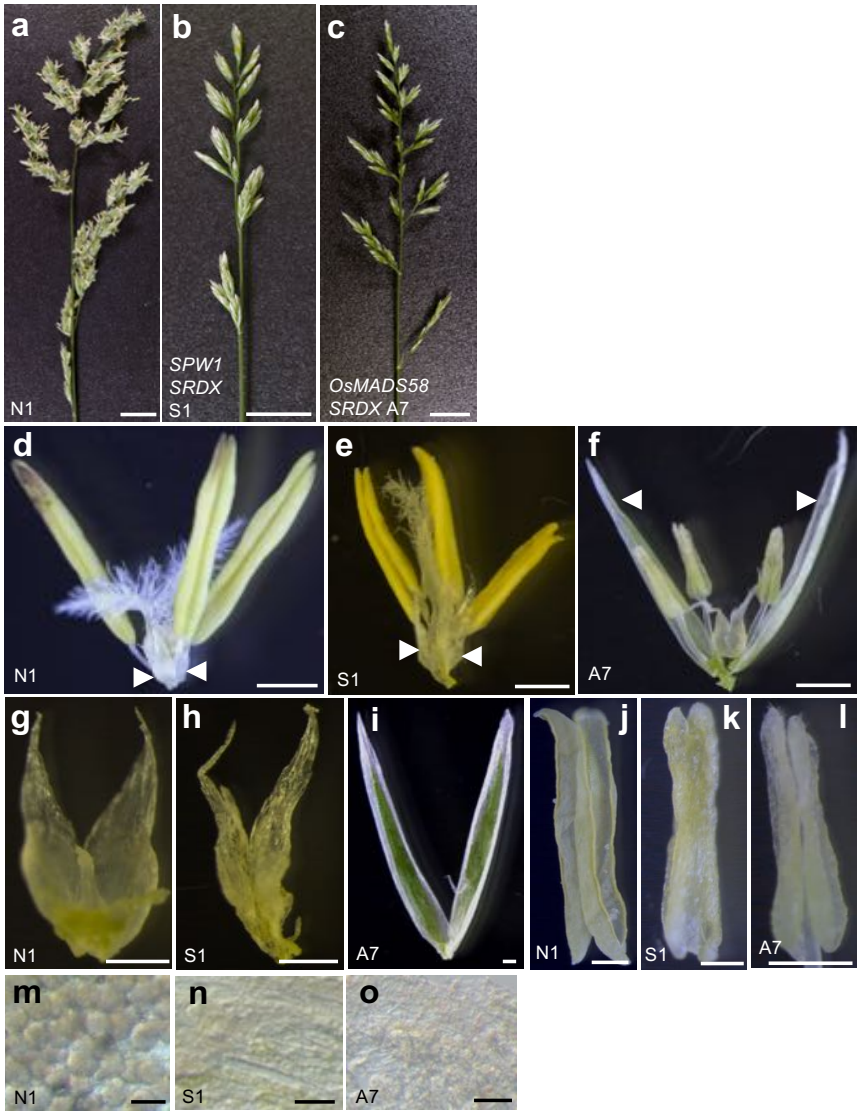

Figure 4.5 Male-sterile and cleistogamous phenotypes in tall fescue expressing *SPW1-SRDX* (b, e, h, k, n) or *OsMADS58-SRDX* (c, f, i, l, o) in comparison with a wild-type plant (a, d, g, j, m). (a–c) Spikelets at the flowering stage. (d–f) Florets with palea and lemma removed. (g) Lodicules (arrowheads in d). (h) Thin and abnormally elongated lodicules (arrowheads in e). (i) Lemma-like organs derived from lodicules (arrowheads in f). (j–l) Anthers. (m–o) Pollen stained with 1% carmine in 45% acetic acid. Bars indicate 1 cm (a–c), 1 mm (d–f), 0.25 mm (g–i), 0.5 mm (j–l) and 50 μm (m–o).

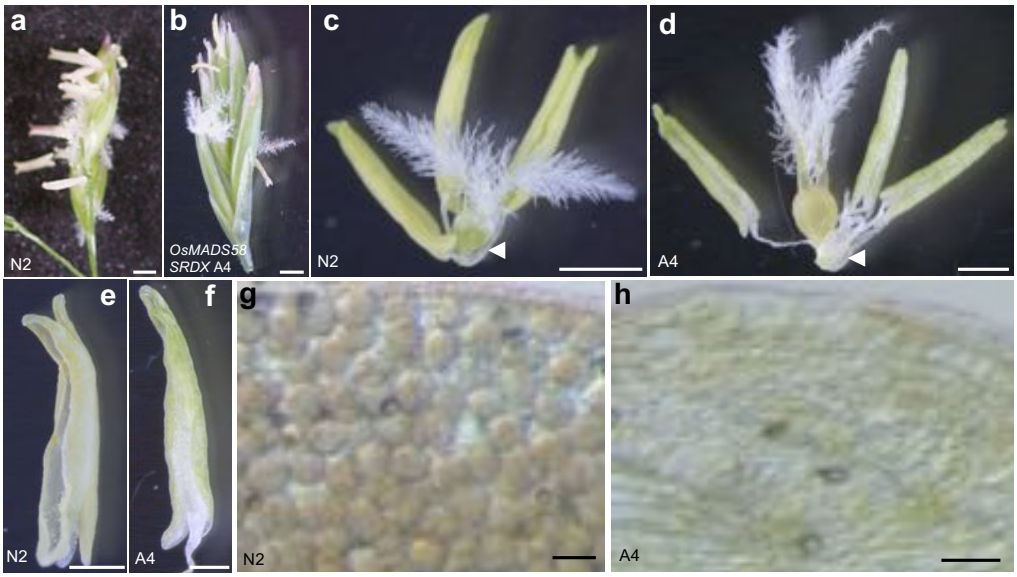

Figure 4.6 Male-sterile phenotype in tall fescue expressing *OsMADS58-SRDX* (b, d, f, h) in comparison with a wild-type plant (a, c, e, g). (a, b) Florets at the flowering stage. (c, d) Florets with palea and lemma removed. Arrowheads indicate lodicules. (e, f) Anthers. (g, h) Pollen stained with 1% carmine in 45% acetic acid. Bars indicate 1 mm (a–d), 0.5 mm (e, f) and 50 μm (g, h).

Chapter 5: General discussion

5.1: Breeding strategies based on transgenic approaches in tall fescue

Transgenic approaches create desired phenotypes and novel genetic variants through the direct introduction of useful genes into target plants. Most genotypes used for genetic transformation are selected on the basis of response in tissue culture rather than agronomic performance. In many cases, transgenes are then incorporated into non-transgenic elite genotypes with agronomically superior traits by backcrossing to exchange the genetic background (Visarada et al. 2009). In outcrossing grasses, it is essential to backcross with a number of elite genotypes to avoid inbreeding repression (Badenhorst et al. 2016). A 'clone and strain synthesis' approach, which exploits both general combine ability and specific combining ability, has been developed to capture heterosis in timothy breeding (Tamaki et al. 2009). The use of elite genotypes selected for this method could lead to higher yield performance. Backcrossing can also eliminate somaclonal variation, which has negative agronomic effects in initial transgenic plants (Dale and McPartlan 1992). Genotypes carrying a single copy of a transgene must be screened in each generation because the heterozygous transgene will be present in only 50% of the progeny (Badenhorst et al. 2016). In commercially available GM alfalfa, each of two independent transgenic lines containing a single transgene was backcrossed with some genotypes of an elite cultivar (Rogan and Fitzpatrick 2004). Each population was crossed to generate a synthetic cultivar with 95% resistance to glyphosate (Rogan and Fitzpatrick 2004). A breeding scheme for the production of a transgenic synthetic cultivar in tall fescue developed on the basis of the alfalfa breeding scheme (Rogan and Fitzpatrick 2004) is shown in Figure 5. In brief, two transgenic plants with either transgene A or B integrated into an independent locus of other genome sets will be crossed with non-transgenic elite genotypes. In each population, plants are heterozygous for the transgene. These populations will be crossed to generate synthetic generation (Syn) 0 plants; the progeny will segregate approximately as follows: 1/4 will carry transgene A; 1/4 will carry transgene B; 1/4 will carry both transgenes; 1/4 will be null. Genotypes with both transgenes will be selected and intercrossed to produce a Syn 1 population (≥ 90% carrying transgene A and/or B). The genotypic frequencies should not shift significantly through

successive synthetic generations (Syn 1, Syn 2 and Syn 3).

5.2: New plant breeding techniques for public acceptance as alternative transgenic approaches

Before the release of GM plants with artificial gene constructs derived from different organisms, risk assessment must be conducted to confirm that they have no adverse effects on food and feed safety and biological diversity (MOE 2018). Although no health hazards of GM food are known, consumers, especially in Europe and Japan, still have concerns associated with health risks (Komoto et al. 2016). Globally, about 70% to 90% of GM crops is consumed by livestock (Flachowsky et al. 2012). When health and performance of GM-fed livestock were closely monitored in numerous experimental studies, they were similar to those of livestock fed non-GM crops (Van Eenennaam and Young 2014). Public concerns about transgenic grasses have focused on the environmental or ecological impacts of pollen-mediated transgene flow because human consumption is indirect (Wang and Brummer 2012).

To address these concerns, cisgenesis was developed as an alternative transgenic approach. In this concept, a transgene introduced into plants by *Agrobacterium*-mediated transformation or particle bombardment consists of a complete gene including introns, promoter and terminator originated from the target species (Schouten and Jacobsen 2008). Selectable marker genes and vector-backbone sequences should be absent or eliminated from cisgenic plants (Holme et al. 2013). Consequently, cisgenic plants are closer to plants generated by conventional breeding. This approach is thought to be the first step toward deregulation of transgenic grasses (Wang and Brummer 2012).

Recent development of genome editing technology using clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) has facilitated plant improvement without integration of transgenes in the final products. While cleavage of a target gene induced by the RNA-guided Cas9 is repaired by an innate DNA repair mechanism, either one or a few nucleotide changes (deletion, insertion or substitution) frequently occur, resulting in gene knockout or alteration (Tang and Tang 2017). At much lower frequency, the CRISPR/Cas9 system can induce a point mutation or gene insertion via homologous recombination based on an externally supplied DNA template (Ahmad and Mukhtar 2017). In traditional genetic transformation, random transgene integration often

disrupts endogenous genes and has a negative effect on plant growth. The CRISPR/Cas9 system allows the insertion, removal or replacement of DNA at particular positions in the genome, avoiding the such deleterious effects. The CRISPR/Cas9 transgene, which is most likely inserted at a separate location to the target gene, can be segregated out in the progeny by crossing with wild-type plants. The CRISPR/Cas9 system has been successfully applied in various plants such as grain crops, fruit trees, *Populus* and alfalfa (Tang and Tang 2017).

Cisgenesis and genome editing are referred to as NPBT (Cardi 2016). Introduction of useful genes isolated from the host species and the absence of foreign DNA should increase consumer acceptance of GM plants developed with NPBT because they might be considered as non-transgenic genetically altered plants (Kamthan et al. 2016). Globally, it has been estimated that the regulatory approval of GM plants costs $35 million and takes six years on average (Phillips McDougall 2011). Many countries including Japan have discussed how to regulate plants modified by genome editing (Ledford 2016). In the USA, USDA announced that it does not regulate genome-edited crops (USDA 2018). Some countries are now moving towards a case-by-case consideration process (Ledford 2016). The EU continues to include genome-edited plants under the GM organism umbrella in the current policy and guidelines (Spicer and Molnar 2018).

5.3: Applications of breeding strategies in tall fescue

The prerequisite for the use of transgenic approaches is the isolation and characterization of genes of interest. The genomic information for cool-season grasses is poor in comparison with that for model plants, but recent advances of genomics and bioinformatics could assist transgenic approaches in grasses. Although only a limited number of potentially useful genes derived from the model plant rice have been tested in tall fescue, they could be evaluated effectively by using developed transgenic approaches; these approaches could also be applicable to improvement of other cool-season grasses. Next, the mutated *ALS* gene tested in Chapter 3 could be used to produce cisgenic plants. Because some amino acid substitutions that confer herbicide resistance are well conserved in plant ALSs (Kawai et al. 2007), HR ALS genes can be developed using this information. Although the use of the CRISPR/Cas9 system has

not been reported in cool-season grasses including tall fescue, genome editing could allow the knockout of TF genes in grasses to improve important traits, such as forage digestibility and male sterility examined in Chapters 2 and 4. In comparison with the severe (cleistogamous) phenotype of *OsMADS58-SRDX* plants, the mild phenotype (only male sterility) caused by partial repression may be available for practical application (Chapter 4). Since point mutations in target genes can lead to mild phenotypes, genomic editing is a promising tool. The efficiency of genome editing is affected by many factors, including vector constructs, gene expression cassettes, target sites, target plants and transformation methods (Bortesi and Fischer 2015; Ma et al. 2016). In addition, it relies on genetic transformation via tissue culture, which is a bottleneck in non-model plants (Altpeter et al. 2016). Transformation efficiency of tall fescue is still not enough to generate sufficient numbers of genome-edited plants. It will be necessary to refine the grass transformation system for generating large numbers of transgenic plants.

In the near future, climate change will impact pasture production and turf maintenance. Demand for livestock products is growing due to the growth of the world population. For stable cultivation and high production in grasslands, it is essential to quickly and effectively improve various agronomical traits such as yield, quality, disease resistance, and tolerance to a wide range of environmental stresses. The transgenic approaches developed in this study for tall fescue could be used for functional analysis of transgenes and to create novel genetic variants useful as breeding materials; the NPBT will allow to accelerate the practical application of transgenic tall fescue.

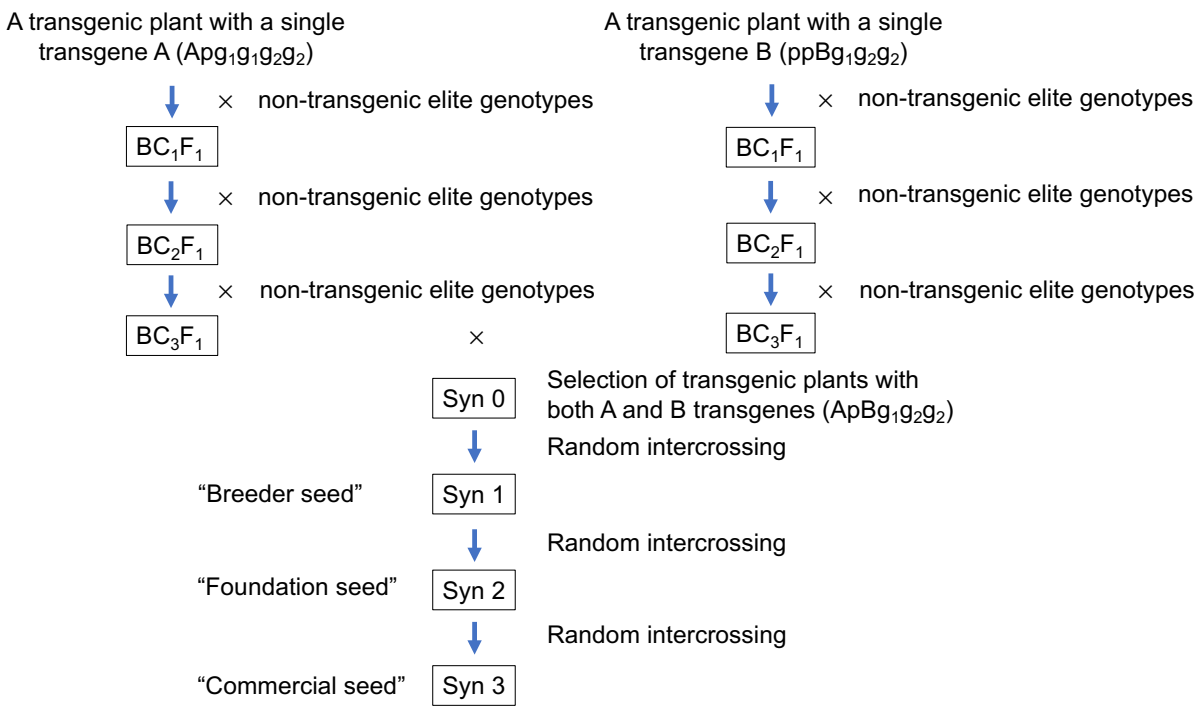

Figure 5 A breeding scheme for the production of a transgenic synthetic cultivar in tall fescue. $Apg_1g_1g_2g_2$, dominant transgene A and null condition on chromosomes; $ppBg_1g_2g_2$, dominant transgene B and null condition on chromosomes; BC, backcross; Syn, synthetic generation.

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Evaluation of transgenic tall fescue (*Festuca arundinacea* Schreb.) improved for important forage and turf traits

Summary

Tall fescue is a major cool-season perennial grasses widely grown for forage, turf and roadside cover. It is tolerant to environmental stresses and is highly persistent. Because tall fescue is an allohexaploid (2n = 6*x* = 42) outcrossing species with self-incompatibility, conventional breeding is time-consuming and genetic improvement is slow. Genetic transformation allows the direct introduction of agronomically useful genes into target plants and the down- or up-regulation of endogenous genes to create desired phenotypes and novel genetic variants. To complement conventional breeding and facilitate improvement of tall fescue, genetic transformation has been used to improve agronomically important traits. Forage digestibility of tall fescue is often lower than that of other cool-season grasses. Because this trait has a direct effect on livestock performance, its improvement is an important target in breeding. Mutants or transgenic plants generated by mutating or down-regulating the genes encoding lignin biosynthetic enzymes have reduced lignin content and increased forage digestibility, indicating that forage digestibility is negatively correlated with lignin content. In turfgrass, herbicide resistance facilitates weed elimination. Transgenic creeping bentgrass and Kentucky bluegrass resistant to glyphosate are already deregulated or their deregulation is pending. However, tall fescue is an anemophilous species and produces large amounts of pollen containing allergenic proteins that cause hay fever in susceptible people. Public concerns about transgenic grasses have focused on the environmental and ecological impacts of pollen-mediated transgene flow. Male sterility is a possible approach to prevent pollen flow into the environment. In this study, transgenic tall fescue was produced with the objective of improving important forage and turf traits, namely forage digestibility, herbicide resistance and male sterility, and the transgene function was evaluated by molecular and phenotypic analyses.

1: Improvement of forage digestibility by *Oryza sativa* SECONDARY WALL NAC DOMAIN2 chimeric repressor (Chapter 2)

The lower forage digestibility in tall fescue is due in part to fiber-constituting secondary cell walls in sclerenchyma cells, which have high cellulose and lignin contents. Thus, sclerenchyma cells can be an important target to improve forage digestibility by genetic manipulation. In rice (*Oryza sativa* L.), the SECONDARY WALL NAC DOMAIN PROTEIN2 (OsSWN2) chimeric repressor under the control of the *OsSWN1* promoter reduces secondary cell wall thickening in sclerenchyma cells. In this study, the *OsSWN2* chimeric repressor was introduced into tall fescue to improve forage digestibility. Of 23 transgenic plants, nine had brittle internodes that were easily broken by bending. The secondary cell walls of brittle plants were significantly thinner than those of the wild-type in the interfascicular fibers of internodes and in cortical fiber cells located between leaf epidermal cells and vascular bundles. The dry matter digestibility increased by 11.8% in stems and by 6.8% in leaves compared with wild-type plants. Acid detergent fiber was reduced by 25.2% in stems and by 21.6% in leaves relative to the mean of wild-type plants. The content of acid-insoluble lignin was reduced in some plants. In phloroglucinol staining to detect lignin distribution, the interfascicular fibers of internodes and cortical fiber cells in leaf veins were less densely stained in *OsSWN2-SRDX* than wild-type plants. Therefore, reduction of secondary cell wall thickness in sclerenchyma cells can decrease indigestible fiber, increasing forage digestibility.

2: Production of herbicide-resistant tall fescue by introducing a mutated *Oryza sativa* acetolactate synthase gene (Chapter 3)

Pyrimidinylcarboxylate (PC) herbicides inhibit acetolactate synthase (ALS) in the biosynthetic pathway that leads to branched-chain amino acids. Mammals have no ALS, and the herbicides are not toxic to them. A rice *ALS* gene with a single point mutation [*OsALS* (sm)] can confer resistance to pyriminobac (PM), a PC herbicide; this gene was used to produce herbicide-resistant tall fescue. Because of the high sensitivity of tall fescue calluses to PM, transgenic calluses were selected by incubation with PM by using the *OsALS* (sm) gene as a selectable marker. Integration of the *OsALS* (sm) gene was confirmed

by Southern blot analysis in ten regenerated plants. The *OsALS* (sm) plants sprayed with PM were unaffected, whereas wild-type plants stopped growing and eventually some parts of each plant died. ALS activity in the leaf tissues of the *OsALS* (sm) plants treated with PM was intermediate between that in wild-type plants with and without PM. These results indicate that the transgenic plants produced OsALS (sm) protein, which conferred PM resistance. One of the transgenic plants was crossed with a wild-type plant. The T_1 plants that inherited the *OsALS* (sm) gene were resistant to PM. Therefore, the phenotype induced by the *OsALS* (sm) gene was stable.

3: Induction of male sterility by chimeric repressors of *SUPERWOMAN1* and *OsMADS58* to prevent pollen flow (Chapter 4)

When an agronomically useful gene and a male-sterile gene reside in the same construct, they will be introduced into plants simultaneously and both improve the target trait and inhibit pollen production. *SUPERWOMAN1* (*SPW1*) and *OsMADS58* specify the formation of stamens in rice. Their loss-of function phenotypes are characterized by homeotic transformation of stamens into other floral organs or other morphological changes, resulting in male sterility. In this study, the chimeric repressors of *SPW1* and *OsMADS58* were evaluated for their abilities to induce male sterility in tall fescue. Although the stamens of transgenic tall fescue were not altered, they produced no pollen or immature pollen; thus, the plants were male-sterile. In addition to male sterility, two *SPW1-SRDX* plants showed a cleistogamous (closed) phenotype in which anthers were not observed outside the glumes, with thin and abnormally elongated lodicules. Five *OsMADS58-SRDX* plants showed a cleistogamous phenotype in which the lodicules were homeotically transformed into lemma-like organs. The phenotype of *OsMADS58-SRDX* tall fescue differed from that of *OsMADS58-SRDX* rice, suggesting functional differences between rice and tall fescue OsMADS58. Two *OsMADS58-SRDX* plants that exhibited only male sterility might be suitable as breeding materials for preventing pollen flow. To use them for this purpose, it is necessary to confirm the stable inheritance in the progeny.

4: Breeding strategies by transgenic approaches in tall fescue (Chapter 5)

Because tall fescue cultivars consist of various genotypes, with each genotype behaving differently in tissue culture, most genotypes used for genetic transformation are selected on the basis of their tissue culture response rather than agronomic performance. In outcrossing grasses, it is necessary to incorporate transgenes into non-transgenic elite genotypes with agronomically superior traits by backcrossing for practical application. Recently, cisgenesis and genome editing have been developed as new plant breeding techniques (NPBT). Cisgenic plants are closer to plants generated by conventional breeding because the transgene consists of a complete DNA copy of a natural gene derived from the same species. The transgene for genome editing, which is most likely inserted at a separate location to the target gene, can be segregated out in the progeny by crossing with wild-type plants. The introduction of useful genes isolated from host species and the absence of foreign DNA is expected to increase consumer acceptance of novel GM plants developed with these techniques. The transgenic approaches developed in this study for tall fescue could be used for functional analysis of transgenes to create novel genetic variants useful as breeding materials; the NPBT will allow to accelerate the practical application of transgenic tall fescue.

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