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IDENTIFICATION OF AFLP MARKERS LINKED WITH LOW-TEMPERATURE RESISTANCE IN INTROGRESSIONS TRANSFERRED FROM *FESTUCA ARUNDINACEA* TO *LOLIUM MULTIFLORUM*

ABSTRACT

BC_3-152/53 population of *L. multiflorum* plants comprising single introgression of *F. arundinacea* genome had higher winter hardiness than control *L. multiflorum* plants. AFLP analysis were performed resulting in generation of 19 markers linked with freezing resistance, 7 linked with winter hardiness and 2 markers correlated with both traits. It indicates that *Festuca* introgression could make the impact on *Lolium* stress resistance.

Key words: AFLP markers, freezing resistance, *F. arundinacea*, *L. multiflorum,* winter hardiness

INTRODUCTION

Low temperature is one of the most important factors that limit the growth and geographical incidence of many grass species. In over-wintering plants from temperate regions, exposing them to certain periods of sub-zero temperature increases their tolerance to latter freezing. Cold or frost acclimation is the adaptive process involving a number of biochemical and physiological changes (Levitt 1980, Guy *et al.* 1985). It also includes the expression of cold-regulated (*COR*) genes, which products are necessary for protection against freezing stress (Thomashow 1999). Winter hardiness is the plant ability to survive in low temperature during winter period. This is a genotype specific trait composed of several components whose impact varies with the climatic conditions (Larsen 1994). Components of winter hardiness include vernalization requirement, photoperiod response, ice encasement, water-logging, desiccation, climatic adaptation, plant condition in autumn, carbohydrate starvation, snow molds, low temperature fungi resistance, and freezing resistance (Larsen 1994).

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In Europe perennial grassland occupy more agricultural areas than any other crop. *Lolium-Festuca* complex is composed of two genus having complementary characters. Within *Lolium* genus two species: *L. perenne* and *L. multiflorum* are economically the most important forage grasses in temperate regions, with high productivity and quality but rather poor persistency. The genus *Festuca* consists of species geographically diverse and better adapted to a wide range of ecological conditions. *Lolium* and *Festuca* species hybridize readily, their homologous chromosomes pair and recombine at high frequency during meiosis (Jauhar 1975, King *et al.* 1999, Naganowska *et al.* 2001, Kosmala *et al.* 2006). Fertile hybrids are commonly utilized to improve *Lolium* cultivars abiotic stress resistance, with maintenance of their good forage quality, by transfer of limited number of *Festuca* genes (e.g. Humphreys *et al.* 1989, Humphreys *et al.* 2005, King *et al.* 1998, Zwierzykowski *et al.* 1998a, b, 1999).

Festuca chromosome segments introgressed into the recipient *Lolium* species can be identified by genomic *in situ* hybridisation (GISH) (Humphreys and Pađakinskienë 1996, Humphreys *et al.* 1997, Kosmala *et al.* 2006, 2007). By a combined approach of GISH technique and amplified fragment length polymorphism (AFLP) it is possible to 'tag' genes responsible for desirable agronomic traits in breeding programmes (Armstead *et al.* 2001, King *et al.* 1998, 2002, Humphreys *et al.* 2005). AFLP technique is a robust and highly effective method for DNA fingerprinting that provides a large number of reliable and reproducible genetic markers that can be used as an alternative to morphological trait analysis. Its application requires no prior sequence knowledge thus is convenient for species in which the genetic information is still limited, like forage grasses.

This paper demonstrates AFLP analysis of diploid *L. multiflorum* population of introgressive forms with single *F. arundinacea* segment. Pentaploid hybrids between hexaploid *F. arundinacea* cv. Kord and diploid *L. multiflorum* cv. Tur were backcrossed into *L. multiflorum* (2x). Obtained BC_3-152/53 population was characterized by freezing resistance and winter hardiness. The objective of the research was generation of markers linked with these both traits.

MATERIAL AND METHODS

Plant material

The pentaploid F_1 hybrids of *F. arundinacea* (2n = 6x = 42) × *L. multiflorum* (2n = 4x = 28) used as the male parent were backcrossed two times into diploid *L. multiflorum* cv. Tur used as the female parent. The introgressions from *F. arundinacea* genome were identified in obtained progenies through GISH. BC_2-152/37/73 plant having single *F. arundinacea* segment (Kosmala *et al.* 2003, 2007) was backcrossed again into *L. multiflorum* to produce BC_3-152/53 population containing 80 plants.

Test for freezing tolerance

Eighty plants of the BC_3-152/53 population were screened for their sensibility to freezing temperatures. Test was performed in controlled conditions with the method described by Rapacz *et al.* (2004).

Each individual was divided into 3 equal-sized clones and established in sand: peat (1:1) mixture in the optimal growth conditions (25°C, 10/14 h day/night photoperiod, 200 $mol \times m^{-2} \times s$ photosynthetic photon flux density (PPFD, Philips AGRO sodium light source, Philips Lightning NV, Turnhout, Belgium)). For 7 day pre-hardening well-rooted plants were transferred to the environmental chamber with following conditions: 12°C, 10/14 h day/night photoperiod, 200 $mol \times m^{-2} \times s^{-1}$ PPFD. Then plants were cold acclimated in sub-zero temperatures (2°C, 10/14 h day/night photoperiod, 200 $mol \times m^{-2} \times s^{-1}$ PPFD). Three weeks later they were put to the stress conditions (-2°C, 10/14 h day/night photoperiod, 200 $mol \times m^{-2} \times s^{-1}$ PPFD) for 1 day. After that time temperature was lowered with the cooling rate 1°C/h. Plants were exposed in -7, -11 and -14°C respectively for 8 hours. Next, they were again transferred to 2°C, 10/14 h day/night photoperiod, 200 $mol \times m^{-2} \times s^{-1}$ PPFD. Defrosting plant tillers were cut down to height of 2 – 3 cm. Then, temperature was raised to 12°C letting plants re-growth. Regeneration of plants was estimated using 0 – 9 visual score (Larsen 1978) where 0 represented dead plants without any signs of re-growth, whereas 9 represented plants without visual signs of damages. Mean scores for 3 replicates of plant were calculated. Statistical significance for differences in freezing resistance between plants was determined using Duncan's multiple range test at $P = 0.05$.

Test for winter hardiness

Field test for winter hardiness for 80 plants of the BC_3-152/53 population were performed during winter period 2005/2006 in Szelejewo Plant Breeding. Winter survival and spring regrowth were scored visually using a scale of 0-9, where 0 represented plants with no visible green tillers and 9 plants without visible winter damage. Estimation was conducted on 16th November 2006 and 15th March 2006. The mean results for 3 clones of each plant from population and control *L. multiflorum* cv. Tur were calculated. The statistical significance for differences between genotypes was determined using Duncan's multiple range test at $P = 0.05$.

AFLP analysis

Genomic DNA was extracted by CTAB method (Murray and Thompson 1980) and purified by phenol extraction. AFLP fingerprints were generated using protocol of Vos *et al.* (1995) with modifications by Kosmala (2004). AFLP adapters consisted of a core sequence and an enzyme-specific sequence (*Eco*RI or *Tru*9I), whereas AFLP primers included a core sequence, specific sequence and a selective extension with one (preamplification) or tree (second amplifi-

cation) selective nucleotides. These primers were used previously for mapping introgressions of winter hardiness and drought tolerance in *L. multiflorum* introgressive lines obtained from backcrosses of triploid and pentaploid *L. multiflorum* × *F. pratensis* hybrids (Skibińska *et al.* 2002).

Polymorphic DNA bands of *F. arundinacea* present in genomes of introgressive genotypes were scored as a dominant (present: absent). Linkage of AFLP markers with freezing resistance or winter hardiness was estimated using analysis of regression (Hastie and Tibshirani 1990).

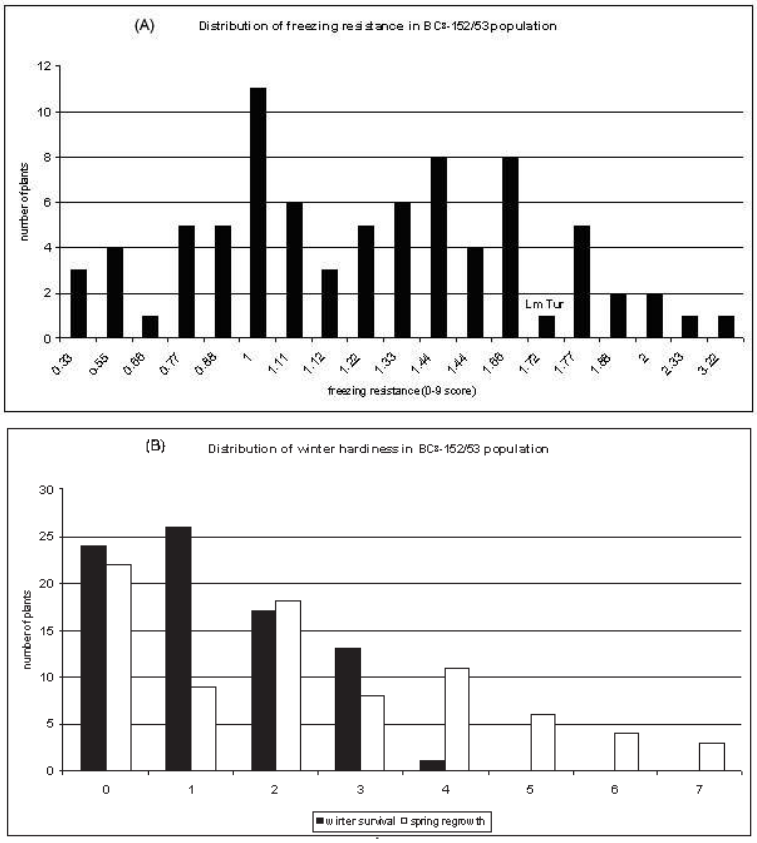

Fig. 1. Distribution of freezing resistance (A) and winter hardiness (B) in BC_3-152/53 population. Both values was assessed on a scale of 0-9 (Larsen 1978), where 0 represented dead plants without any signs of re-growth and 9 plants without visual signs of damages. Control (*L. multiflorum* cv. Tur) was marked as Lm Tur — histogram (A).

RESULTS

The diversity of freezing sensibility and winter hardiness in BC_3-152/53 plants was illustrated by histograms (Fig. 1a, b). Freezing tolerance of 11 individuals was significantly ($P < 0.05$) higher (1.78-3.22) than the mean value of

L. multiflorum (1.72). Winter hardiness of 13 plants was better ($P < 0.05$) than control *L. multiflorum*.

AFLP studies were performed on three groups of plants. BC_3-152/53-A group contained 10 freezing-sensitive and 10 freezing-tolerant individuals. To BC_3-152/53-B 10 plants winter hardy and 10 with low hardiness after winter were selected. The third group (BC_3-152/53-C) consisted with 10 plants having both high freezing resistance and winter hardiness values and 10 plants with the lowest freezing resistance and winter hardiness values. Forty primer combinations were used for the identification of introgressions transferred from *F. arundinacea* to *L. multiflorum* genome. The total number of AFLP markers obtained was 28. Nineteen of them were linked with freezing resistance (BC_3-152/53-A), 7 with winter hardiness (BC_3-152/53-B) and 2 markers were simultaneously correlated with both analysed traits (BC_3-152/53-C). Their approximate size (in base pairs) ranged from 130 to 700. Primer pairs giving the highest number of polymorphic bands derived from *F. arundinacea* in respective analysed groups of plants were following: BC_3-152/53-A - E43M47 (23), BC_3-152/53-B - E32M33 (26), BC_3-152/53-C - E43M52 (21).

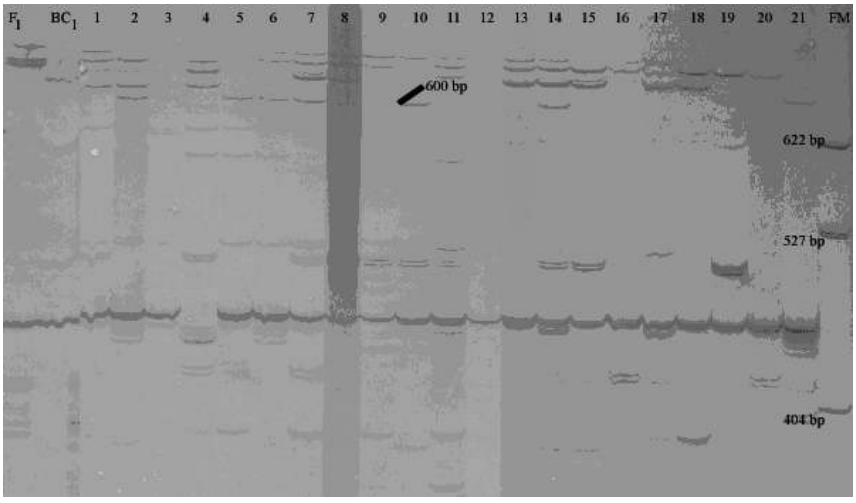

Fig. 2. AFLP fingerprint obtained by DNA amplification with E43M52 primer combination, separated in 6% denaturing acrylamide gel and visualised with silver staining. Individual genotypes from BC_3-152/53 population are marked with numbers, and length marker with M letter. Arrow shows DNA band being marker linked with freezing resistance.

DISCUSSION

Species from *Festuca* genus are the excellent source of low-temperature stress tolerance. Among them *F. pratensis* is the most freezing-resistant and winter hardy cultivated in Europe. Additionally its diploid nature causes that introgression of stress resistance proceeds relatively easily. Transfer of freezing tolerance from *F. pratensis* to *L. perenne* was reported previously (e.g. Canter 2000, Grřnnerřd *et al.* 2004).

F. arundinacea is allohexaploid species comprising three subgenomes, one inherited from freezing tolerant *F. pratensis* and two from drought tolerant *F. glaucescens* (Humphreys *et al.* 1995). It gives two approaches for transfer of stress resistance genes to *Lolium*: indirectly from the subgenomes (Humphreys and Thomas 1993, Humphreys and Pađakinskienë 1996, Humphreys *et al.* 1997) or directly from one or the other of its progenitors (Zwierzykowski *et al.* 1999, Morgan *et al.* 2001, Humphreys *et al.* 2005, Kosmala *et al.* 2006). The first backcross-breeding program aimed at the transfer of winter hardiness and freezing tolerance from *F. arundinacea* into *L. multiflorum* using pentaploid hybrids between *F. arundinacea* (6x) and *L. multiflorum* (4x) was reported by Kosmala *et al.* (2006). The current study, is the second backcross-breeding program with the utilization of these hybrid performed on different *L. multiflorum* cultivars.

Among BC_3-152/53 population 13.8% and 16.25% individuals showed higher than paternal *L. multiflorum* plants values of freezing resistance and winter hardiness, respectively. In Kosmala *et al.* (2006) research only 7.2% of the tested genotypes showed more winter hardiness then the *L. multiflorum* control. Simultaneously all these plants were more freezing tolerant than *Lolium* and the level of resistance varied significantly (from 0.5 to 1.5). In our experiment AFLP research resulted in only two AFLP-markers linked with both traits generation. Although freezing tolerance is the most important component of winter hardiness the final contribution of freezing tolerance in winter hardiness is very variable and strongly depends on the year and local conditions (Larsen 1994). Moreover, it was noticed that correlation between freezing tolerance and winter hardiness varies clearly among species. In wheat (Sutka *et al.* 1986) and oilseed rape (Rife and Salgado 1996) frost resistance is in general well correlated with winter hardiness. But there are species, like alfalfa, where this correlation is rather low, depending of the year of observations (Brouwer *et al.* 2000). In our experiment we obtained only two markers connected with both traits. In our experiment conducted in Szelejewo Plant Breeding meteorology data were monitored. Winters in 2005 and 2006 were rather warm, with low number of day with the negative temperatures operating to the plant. It is supposed that under such mild conditions, freezing tolerance could not be a factor determining winter hardiness of the plants. It can not be also excluded that *F. arundinacea* belongs to the species characterizing with week genetically-based relationship between frost resistance and winter hardiness but it needs a further experimental verification.

CONCLUSIONS

To recapitulate, it was shown for the second time that utilization of *F. arundinacea* in backcross breeding approaches takes the opportunity to transfer genes for low-temperature resistance to *L. multiflorum*. In BC_3-152/53 population introgressions were indicated by AFLP analysis. With the assistance of

primers used in the present experiment it was possible to distinguish chromosome regions separately responsible for winter hardiness and freezing tolerance. *Festuca* introgression could make the impact on *Lolium* winter hardiness and freezing resistance as some individuals from BC_3-152/53 population were characterized by higher stress tolerance than paternal *L. multiflorum* plants.

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