Efficient Doubled Haploid Production in Perennial Ryegrass (*Lolium perenne* L.)

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R.F. Begheyn, K. Vangsgaard, N. Roulund, and B. Studer

Abstract

Hybrid breeding has contributed significantly to the enormous yield increases that many major crops have undergone during the previous century. Its success relies on the exploitation of heterosis, the superior performance of an F1 hybrid compared to its inbred parents. Attempts to implement hybrid breeding in forage grasses, such as perennial ryegrass (*Lolium perenne* L.), are hampered by its highly effective self-incompatibility system as well as its sensitivity to inbreeding depression. Homozygous inbred lines are therefore difficult to create using the classical method of repeated selfing. Here, we report an efficient method to obtain homozygous genotypes of perennial ryegrass using doubled haploid (DH) induction. By means of anther culture, completely homozygous lines were obtained within one generation cycle. A highly genotype dependent response was observed for traits such as the number of embryos/calli per 100 cultured anthers and the percentages of green and albino plants regenerated. Transgressive segregation, indicative of heritable and polygenic control of the traits, was also found. Our general aim is to develop a molecular marker system to select for high responsiveness and to facilitate the introgression of this trait into advanced breeding germplasm. Segregating mapping populations will be phenotyped during anther culture and genotyped via a genotyping-by-sequencing (GBS) approach. Family-based association mapping will be used to identify marker-trait associations. In this way, an efficient breeding tool to screen germplasm for DH induction capacity will be developed. Our work will significantly accelerate forage grass breeding and constitute the first step towards efficient production of grass hybrids.

R.F. Begheyn (✉) • B. Studer
Forage Crop Genetics Institute of Agricultural Sciences, Forage Crop Genetics, ETH Zurich, Zurich, Switzerland
e-mail: rachel.begheyn@usys.ethz.ch

K. Vangsgaard • N. Roulund
DLF Trifolium A/S, Højerupvej 31, Store Heddinge DK-4660, Denmark

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Keywords

Hybrid breeding • Doubled haploids • Androgenesis • Molecular markers • Breeding tool • Perennial ryegrass

Introduction

To maintain and improve the productivity and sustainability of grasslands, novel tools and innovative breeding strategies are needed (Smith and Spangenberg 2014). The effective method of hybrid breeding can currently not be applied to forage grasses such as perennial ryegrass (*Lolium perenne* L.), because the self-incompatibility (SI) system of this species hampers the development of homozygous lines. Usually created by repeated selfing, inbred lines are used by breeders as parents to generate F1 hybrids. The best performing offspring of all tested parental combinations may then be released as a new cultivar. Hybrids often display hybrid vigor (heterosis), enabling them to outperform their parents for important agronomical traits (Shull 1948). This phenomenon has also been documented in perennial ryegrass (Posselt 2010). Implementing hybrid breeding therefore has great potential to contribute to the acceleration of forage grass breeding.

Doubled haploid (DH) induction is an efficient way to achieve 100 % homozygosity in one generation. This method not only bypasses the obstacle of SI, but also that of inbreeding depression, which increases in severity with every additional cycle of selfing. DH production in perennial ryegrass through anther culture has been reported (Olesen et al. 1988). However, the response to androgenesis, for example the percentage of albinos obtained, is highly variable and depends on the genotype used (Madsen et al. 1995). Nevertheless, androgenetic capacity has successfully been transferred to less responsive material by crossing it with so-called inducers, extremely rare genotypes that respond well to DH induction (Halberg et al. 1990; Madsen et al. 1995). Although the genetics underlying androgenetic capacity have not been studied in detail in perennial ryegrass, it is generally thought that a limited number of recessive genes is responsible (Madsen et al. 1995).

Here we report the results of a pilot experiment in which we implemented an efficient DH induction method in perennial ryegrass and phenotyped a segregating population for androgenetic capacity. The next step will be to apply the DH induction protocol to a set of segregating mapping populations, which will also be genotyped using a genotyping-by-sequencing (GBS) approach (Elshire et al. 2011). Family-based association mapping can then be used to identify marker-trait associations for traits of importance in adrogenesis (Guo et al. 2013).

Our general aim is to develop a molecular marker based breeding tool to select for high responsiveness to anther culture and to facilitate the introgression of this trait into advanced breeding germplasm. Research on forage grass cytoplasmic male sterility (CMS) has recently shown significant progress (Studer et al. 2012; Islam et al. 2014). Practical and economical systems for pollination control as well as homozygous line production are now within reach. Our work will therefore significantly accelerate forage grass breeding and will constitute the first step towards the realization of hybrid grass cultivars.

Material and Methods

Plant Material

Three so-called inducer genotypes, known to exhibit a good response to DH induction, were used. Offspring of a polycross between a larger set of inducer genotypes, including the three used in this experiment, was also made available to us and we used the eighteen genotypes that flowered at the convenient time. The plants were vernalized and grown in an unheated greenhouse in 2013/2014. Spikes with microspores in the late uninucleate stage, as determined by microscopy, were harvested in May-June 2014 for use in the doubled haploid induction experiment.

Doubled Haploid Induction

The method used was adapted from the androgenesis protocols based on 190–2 media for perennial ryegrass described by Olesen et al. (1988) and Opsahl-Ferstad et al. (1994). Kinetin and 2,4-dichlorophenoxyacetic acid (2,4-D) were included in the induction medium. Kinetin and 1-naphthaleneacetic acid (NAA) were included in the regeneration medium, but the shoot rooting medium did not contain any hormones. Maltose instead of sucrose was used as the carbon source in the induction medium. The gelling agent was Gelrite at 3.5 g L^{-1}. A cold pre-treatment of the spikes of 1–5 days was applied. Spikes were sterilized by a quick rinse in 70 % ethanol, followed by a 10-min soak in a 2.5 % hypochlorite solution.

Depending on spike availability, 84–168 anthers of the three inducer and eighteen polycross progeny genotypes were subjected to the *in vitro* DH induction procedure. Component traits of androgenetic capacity, such as the numbers of responsive anthers, embryo-like structures or calli and green or albino plants that were obtained were recorded. A representative subset of regenerated green putative DH plantlets was finally transplanted into soil and grown in the greenhouse.

Flow Cytometry

Leaf material of a subset of the green putative DH plants was harvested to estimate DNA content using flow cytometry. A genotype of the cultivar 'Arara' was used as a diploid control to determine the ploidy level of the putative DH plants.

Results and Discussion

DH Induction

Marked differences in androgenetic capacity were observed between the genotypes for the number of embryo-like structures (ELS) and plants produced per 100 anthers as well as for the percentages of albino and green regenerants (Table 23.1). Some genotypes did not produce any ELS, while for other genotypes the ELS did not regenerate into plants. Genotypes that yielded plants showed distinct differences in ratios between albino or green plants. Two genotypes could only be induced to regenerate albinos.

Several of the PP genotypes performed better than the best performing inducer for several or even all recorded traits, as was the case for PP 078. Such transgressive segregation is an indication of polygenic control of the component traits of androgenic capacity, confirming similar reports in literature (Madsen et al. 1995; Wang et al. 2005).

Flow Cytometry

In perennial ryegrass anther culture, the chromosome doubles spontaneously in the early phases of embryogenesis, with a reported efficiency of 50–70 % (Olesen et al. 1988; Bante et al. 1990; Halberg et al. 1990). Our results confirm that spontaneous chromosome doubling takes place. Interestingly, variation between genotypes was observed, suggesting genetic control of this characteristic (Table 23.2). It is also interesting that some genotypes that display similar performances for plantlet regeneration per 100 anthers and percentage of green regenerants differed in the percentages of diploids they yield. PP 007 and PP 012 produced 98 and 114 plants per 100 cultured anthers, respectively, approximately 70 % percent of which were green (Table 23.2). However, PP 007 had a chromosome doubling efficiency of 33 % compared to 50 % for PP 012.

Conclusions

An effective DH induction protocol based on anther culture was successfully established and could be used to screen perennial ryegrass genotypes for DH induction capacity. An intriguing variation for component traits of androgenetic capacity was observed between the genotypes. The ploidy level

Table 23.1 Overview of the number of embryo-like structures (*ELS*) and number of plants regenerated per 100 anthers, as well as the percentages of albino and green plants that were obtained by doubled haploid induction

Genotype	Number of ELS per 100 anthers	Number of plants per 100 anthers	Percentage albino plants	Percentage green plants
Inducer 002	277	173	81	19
Inducer 046	4	0		
Inducer 149	95	0		
PP 006	50	0		
PP 007	297	98	29	71
PP 012	323	114	34	66
PP 015	52	32	70	30
PP 017	24	0		
PP 023	67	32	100	0
PP 024	113	10	69	31
PP 031	23	36	67	33
PP 039	42	15	38	62
PP 046	1	0		
PP 051	0			
PP 056	102	70	41	59
PP 071	5	0		
PP 078	580	349	32	68
PP 079	40	42	29	71
PP 081	30	21	100	0
PP 086	2	0		
PP 089	0			

PP polycross progreny

Table 23.2 Ploidy levels, determined by flow cytometric DNA content estimation, of a subset of the green putative doubled haploid (*DH*) plantlets obtained in the DH induction experiment

Genotype	Number of plants checked	Percentage of haploids (n)	Percentage of diploids (2n)	Percentage of tetraploids (4n)
Inducer 002	11	36	64	
PP 007	9	56	33	11
PP 012	10	30	50	20
PP 015	10		100	
PP 024	1	100		
PP 031	10	10	90	
PP 056	10		100	
PP 078	10	10	70	20
PP 079	10		100	

PP polycross progeny

analysis still needs to be complemented with a marker assay to determine whether the diploid genotypes are actual DH plants. Those results will enable us to draw clear conclusions on the outcome of our experiment. These preliminary results are encouraging for our future work with mapping populations segregating for those same characteristics and inspire confidence that we will be able to find marker–trait associations useful for the development of a novel breeding tool.

Inbreeding depression is problematic when homozygosity is increased in outcrossing species such as perennial ryegrass, leading to DHs that are often weak and suffer from fertility issues (Opsahl-Ferstad 1993). However, reports of DH genotypes with a higher vigor and seed set than their parents do exist (Andersen et al. 1997). In effect, a selection against inbreeding depression takes place while raising DH plants. The fertile and vigorous genotypes are excellent candidates for parents of hybrid grass cultivars.

In conclusion, our future results and the molecular breeding tool we will develop, taken together with the foreseen developments in grass CMS research, will bring us closer to the reality of hybrid grass cultivars than ever before.

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