Inheritance patterns of the response to in vitro doubled haploid induction in perennial ryegrass (*Lolium perenne* L.)

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Abstract The ability to produce doubled haploid (DH) plants has found broad application in research and breeding. For major crop species such as maize (*Zea mays* L.) and barley (*Hordeum vulgare* L.), routine large-scale production of DHs has enabled the acceleration of breeding processes, for example through efficient generation of homozygous lines. However, in forage crops such as perennial ryegrass (*Lolium perenne* L.), low and genotype-specific responses to in vitro anther culture (AC) still limit wide-spread use of DHs. Here, we report the responses of nine bi-parental populations, segregating for microspore embryogenesis and plant regeneration capacity, to an effective AC protocol. Genotypes of exceptionally high androgenic ability, producing over 200 green plants per 100 anthers cultured, could be selected. Continuous and distinctly shaped distributions for the evaluated traits were indicative of quantitative polygenic control and the presence of different alleles in each population. An insignificant association of embryo production with plant regeneration,

as well as a low correlation between green and albino plant yield ($\rho=0.20$), suggested that different genes influence these traits. The populations evaluated here provide a rich source of alleles needed for the introgression of high levels of androgenic capacity into recalcitrant material. Moreover, this germplasm is ideally suited for use in future genotyping and mapping studies so that the genetic control of androgenic capacity in perennial ryegrass can be elucidated. Ultimately, our results will help to realize the potential of DH induction in one of the world's most important forage crop species.

Keywords Androgenic capacity · Androgenesis · Anther culture · Doubled haploid (DH) · Perennial ryegrass (*Lolium perenne* L.)

Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
AC	Anther(s) culture(d)
EC	ELS cultured
ELS	Embryo-like structures
DH	Doubled haploid
GWAS	Genome-wide association study/studies
HRM	High resolution melting
NAA	1-Naphthaleneacetic acid
QTL	Quantitative trait locus/loci
SSR	Simple sequence repeat

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Introduction

The ability to produce doubled haploid (DH) plants significantly increases the efficiency and efficacy of basic and applied research as well as plant breeding programs. Access to completely homozygous material has been

invaluable for the creation of mapping populations for genetic and genomic studies (Forster et al. 2007) and the simplification of genome sequence assembly (Dunwell 2010). Through self-pollination, DH populations can be propagated indefinitely and stored as seed, thus allowing for multi-year and multi-location phenotyping for QTL discovery (Guo et al. 2013), which has been particularly useful in cereal species (Cabral et al. 2014; Sannemann et al. 2015; Obsa et al. 2016). In breeding programs, DH production replaces inbred line development through repeated self-pollination, so that major reductions in the time to cultivar release are achieved (Dwivedi et al. 2015). Many of the modern barley (*Hordeum vulgare* L.), rice (*Oryza sativa* L.) and wheat (*Triticum aestivum* L.) cultivars are DHs (Devaux and Kasha 2009; Niu et al. 2014; Mishra and Rao 2016; Niak et al. 2016). When used as parents in hybrid breeding, DHs allow for effective exploitation of hybrid vigour (heterosis) (Birchler et al. 2010). Doubled haploid techniques have, for example, been essential in bringing about last century's major increases in maize (*Zea mays* L.) yields (Geiger and Gordillo 2009).

Immature microspores are the starting material of choice for in vitro DH induction, since they are usually abundantly available and, by applying a stress treatment, can be induced to develop into embryos and then plants (Seguí-Simarro and Nuez 2008; Ferrie and Caswell 2011). Even though a plethora of anther and microspore culture protocols are available (Maluszynski et al. 2003; Dwivedi et al. 2015), their success is highly species- and genotype-specific (Seguí-Simarro 2010). In practice, access to DH production and its associated benefits is therefore restricted to the few crops in which methods are sufficiently developed to allow routine application, such as barley, tobacco (*Nicotiana tabacum* L.) and rapeseed (*Brassica napus* L.) (Seguí-Simarro 2015). Despite the relatively advanced state of DH techniques in perennial ryegrass (*Lolium perenne* L.), the world's most economically valuable forage crop, they are not yet part of the standard repertoire of researchers and breeders (Arias Aguirre et al. 2011; Pembleton et al. 2015). A self-incompatibility system prevents efficient development of inbred lines in this species (Cornish et al. 1979), so that DH induction through androgenesis may well be the most practical way to obtain homozygous germplasm and thus enable, for instance, hybrid seed production (Begheyne et al. 2016a). Since DH research in perennial ryegrass began in the late 1970s, anther culture (AC) protocols have been successfully adapted from wheat (Olesen et al. 1988) and barley (Boppenmeier et al. 1989), improved by optimizing the sugar source (Bante et al. 1990) and cold pretreatment duration (Opsahl-Ferstad et al. 1994a), and even converted to isolated microspore culture (IMC) (Andersen et al. 1997).

Nevertheless, it was soon realized that high androgenic capacity in perennial ryegrass is not only strongly genotype-dependent, but, similar to wheat, also a rare trait in breeding germplasm (Olesen et al. 1988; Bante et al. 1990; Madsen et al. 1995; Nielsen et al. 2015). In addition to improved induction protocols, introgression of androgenic capacity was therefore deemed a prerequisite for the implementation of DH production in perennial ryegrass breeding programs (Halberg et al. 1990). Investigations into the DH induction responses of genotypes derived from parents with known performances revealed that distinct genetic factors control embryo formation, plant regeneration frequency, and the percentage of green plants (Andersen et al. 1997). The presence of a few recessive genes with large effects, controlling embryo formation in an additive manner and regeneration and green plant frequencies in a dominant manner (Opsahl-Ferstad et al. 1994b), explained the observed genetic interactions. Genotypes exhibiting a high production of embryos and green plants were intercrossed to combine their beneficial alleles and resulted in the selection of genotypes with up to 53.5 fold increases in green plant production (Halberg et al. 1990). These 'inducers' were then used to introgress androgenic capacity into unresponsive material, which resulted in a 7.3 fold increase in green plant production (Madsen et al. 1995).

By the middle of the 1990s, it was supposed that AC would soon find wide application in breeding programs because an in vitro protocol was available and the 'inducer' approach was demonstrated to be effective (Opsahl-Ferstad et al. 1994a). Remarkably, the two decades that followed saw neither scientific communications on DHs in perennial ryegrass nor their use in commercial breeding, possibly due to the time consuming technical and empirical efforts required to achieve methodological improvements (Forster et al. 2007). Significant advances in fields such as genetics, genomics and biotechnology made during that time, resulted in unprecedentedly effective tools to potentially unravel the genetic mechanisms behind DH induction capacity. Moreover, the synergy between many of these technologies and DH techniques, microspore transformation for direct transgene fixation for example (Guo et al. 2009; Schedel et al. 2016), are sufficient reason to believe that there have never been more advantageous and impactful applications of DHs possible in perennial ryegrass research and breeding than there are now (Begheyne et al. 2016a, b).

By using appropriate germplasm in concert with current genotyping and mapping approaches, discovery of the genetic loci involved in androgenic capacity became feasible. For example, a recent genome-wide association study (GWAS) in wheat found two QTL on chromosomes 1B and 7B that explained 53% of the variation in green plant production (Nielsen et al. 2015). Similarly, genes

on chromosome 4A seem to be responsible for total and green plant production in triticale (*×Triticosecale* Wittm.) (Krzewska et al. 2012, 2015). Molecular markers have been developed through such studies, and now enable introgression of androgenic capacity into recalcitrant breeding germplasm. In perennial ryegrass however, the genetic resources to perform this type of investigation have yet to be established, so that a modern reboot of research on the production of DHs can be initiated (Andersen et al. 1997; Begheyn et al. 2016a).

The main objective of this research was to create such a resource by evaluating the response to in vitro AC of different perennial ryegrass populations segregating for androgenic capacity. Specifically, we aimed to investigate whether (1) our AC method was effective in this germplasm, (2) the genetic control of the component traits of androgenic capacity could be elucidated, (3) highly androgenic genotypes could be selected for use in introgression breeding, (4) the regenerated green plantlets were homozygous, diploid and had an acceptable performance in the field, and (5) the studied populations would be suitable for future genotyping and mapping studies.

Materials and methods

Plant material

The plant material used in this study was developed at the research and breeding station of DLF A/S in Store Heddinge, Denmark, and originated from a DH induction program. Paircrosses were made between genotypes with differing responses to in vitro AC. Harvested seeds were sown in autumn 2014, vernalized under natural conditions in an unheated greenhouse over winter. Fifty randomly selected progenies of each paircross were grown in an unheated greenhouse in soil filled 13 cm diameter (1 L pots) during 2015 and 2016 at the experimental research station of ETH Zurich in Eschikon, Lindau, Switzerland. Paircross parents and their offspring populations (anther donors) are numbered according to Fig. 1. The paircross parents capable of producing green plants will hereafter be referred to as being androgenic (P2, P102 and P169) while all others will be referred to as being non-androgenic (Supplementary Table S1).

Haploid and doubled haploid induction

In order to select and prepare donor spikes for in vitro AC, the microspore developmental stage was assessed by harvesting anthers from the middle of a spike for a squash preparation in a drop of water on a glass slide, and observing the cells using a stereomicroscope. Spikes with anthers

Fig. 1 Names and anther culture response characteristics of the paircross parents of the populations (Pop) evaluated in this study

in which the majority of the microspores were in the late-uninucleate stage were harvested and kept at 4 °C in the dark for 24–72 h before being used for AC.

After the cold treatment, spikes were surface sterilized for 1 min in 70% ethanol with a drop of Tween-20, followed by stirring for 10 min in 2.5% sodium hypochlorite with a drop of Tween-20, and four rinses in sterile demineralized water. Adapted versions of the 190-2 medium described by Wang and Hu (1984) were used for all culture steps. In all cases, the $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ concentration was increased to 140 mg L^{-1} , the $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ concentration was decreased to 4.9 mg L^{-1} , the $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ concentration to 13.9 mg L^{-1} , and the $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$ concentration to 18.6 mg L^{-1} . For the induction medium, 1-naphthaleneacetic acid (NAA) was replaced by 1.5 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), and sucrose by 90 g L^{-1} maltose. Hormone concentrations were reduced to 0.25 mg L^{-1} in the regeneration medium and omitted altogether in the resting medium. A concentration of 0.35% Gelrite (Duchefa Biochemie, Haarlem, The Netherlands) was used for solidification and the pH was set to 6. All culture steps took place at 26 °C, with a 16 h photoperiod.

Aseptically excised anthers were placed in a 90 mm Petri dish containing induction medium. A density of 126 anthers per dish, derived from the 7 spikelets in the middle of each spike, was used. Depending on flower availability, between 126 and 252 anthers were cultured for the majority of anther donor genotypes. After 6–8 weeks, ELS were moved onto the regeneration medium for shoot and root induction. Green regenerants were recovered after 3–4 weeks and grown on resting medium in ‘De Wit’ culture tubes (Duchefa Biochemie, Haarlem, The Netherlands) for another 2–3 weeks before transfer to the greenhouse. Anthers of some genotypes produced so many embryos and/or green plants that representative subsamples had to be taken for further culture and analysis.

Phenotypic evaluation of the response to haploid and doubled haploid induction

The following observations were recorded for each anther donor genotype: (1) the percentage of embryogenic anthers, which were those producing macroscopic embryo-like structures (ELS), (2) embryo formation, expressed as the number of ELS per 100 anthers cultured (AC), (3) plant yield, expressed as plant production (number of plants per 100 AC) or plant regeneration (per 100 ELS cultured (EC)), and (4) green plant yield, expressed as green plant production (number of green plants per 100 AC) or green plant regeneration (per 100 EC). The percentages of green and albino regenerants per genotype were also calculated.

Statistical analysis

All analyses were performed and all figures were generated in RStudio (RStudio Team 2015) running R version 3.3.1 (R Core Team 2016) and using the packages ggplot2 (Wickham 2009) and cowplot (Wilke 2016). Rank-based, non-parametric tests (Kruskal–Wallis with Bonferroni corrected Dunn's tests post hoc ($P \leq 0.05$), Wilcoxon–Mann–Whitney tests and Spearman's rank-order correlation tests) were used for the statistical analyses, since the data violated the necessary assumptions for parametric testing and transformation did not offer significant improvement (Manninen 2000).

Genetic characterization of the plant material

DNA was extracted from anther donor plants and green regenerants using a 96-well plate KingFisher Flex Purification System and KingFisher Pure DNA Plant Kits (Thermo Fisher Scientific, Waltham, MA, USA). Simple sequence repeat (SSR) markers G05_065 and G05_134 (Studer et al. 2008) and high resolution melting curve analysis (HRM) marker 05_02833 (Manzanares et al. 2016), selected because of their heterozygosity in a large number of anther donor genotypes, were used to characterize the green regenerants. A detailed primer description and the PCR amplification protocol used for the SSR markers can be found in Studer et al. (2008). Amplification products were separated, visualised and scored using an ABI 3130 16 capillary electrophoresis system (Applied Biosystems, Foster City, CA, USA) and the GeneMarker software version 1.5 (SoftGenetics, PA, USA). HRM analysis was performed as described in Studer et al. (2009) and Manzanares et al. (2016). Briefly, PCR amplicons were melted between 60 and 98 °C using a 96-well LightScanner and genotyping was performed with the LightScanner software package (BioFire Diagnostics, UT, USA).

Leaf tissue samples of the green regenerants were prepared and analysed as described in Doležel et al. (2007), following steps 1C and 2B. Samples were analysed using a CyFlow Space flow cytometer (Sysmex Partec GmbH., Görlitz, Germany) equipped with a UV led diode array. At least 2500 events were acquired per sample and only measurements with a coefficient of variation for the G0/G1 peaks of $<2.5\%$ were accepted.

Results

Phenotypic responses to anther culture

A total of 351 genotypes, derived from nine distinct pair-cross populations, were subjected to in vitro AC during two flowering seasons. Depending on flower availability, between 17 and 50 genotypes could be evaluated per population. A total of 75,726 anthers were cultured, 17.1% of which were embryogenic. On average, the embryo induction rate was 86.7 ELS per 100 AC, the regeneration frequency 76.2 plants per 100 AC (or 53.4 plants per 100 EC) and 38.2 green and 38.0 albino plants were obtained per 100 AC (or 20.1 green and 33.3 albino plants per 100 EC).

In 2015, 74.6% of the 295 evaluated genotypes produced ELS, 56.6% produced plants, 30.1% produced green plants (3.1% did so exclusively) and 26.8% produced only albinos. The genotypes responded differently for the evaluated traits and a wide segregation within each population was observed (Fig. 2; Supplementary Fig. S1). The averages of most populations were close to zero for plants per 100 AC and green plants per 100 AC or EC, with population 11 having the lowest overall performance. Nevertheless, for each evaluated trait, at least several responsive genotypes could be identified within each population. Remarkably embryogenic genotypes were observed in populations 2, 3 and 4 (Fig. 2b), while populations 3, 4 and 7 included genotypes producing high numbers of plants (Fig. 2c). Population 6 included genotypes with a relatively high plant regeneration frequency (Fig. 2d) and green plant regeneration per 100 EC (Fig. 2f), as well as the anther donor from which the most green plants per 100 AC were obtained (Fig. 2e). Several genotypes producing over 100 green regenerants per 100 AC could be identified in all populations, except population 11.

Genotypic differences were observed for the percentages of green plants that were regenerated, both within and between the evaluated populations (Fig. 3). Within populations 1, 7 and 8, no genotypes produced more than 65% green plants for instance, whereas anther donors of population 11 produced either green or albino plantlets exclusively.

Fig. 2 Box plots of the responses of the evaluated populations to anther culture. The letters above each plot denote significant differences between populations ($P \leq 0.05$). **a** Percentage of embryogenic anthers; **b** number of embryo-like structures (ELS) per 100 anthers cultured (AC); **c** number of plants regenerated per 100 AC; **d** number

of plants regenerated per 100 ELS cultured (EC); **e** number of green plants (GP) regenerated per 100 AC; **f** number of GP regenerated per 100 EC. For graphical reasons, observation 1530 from population 6 has been omitted from graph **e**

Fig. 3 Percentages of green plants per genotype per population, regenerated during the 2015 experiments

Highly androgenic genotypes, those producing embryos in large numbers as well as having a very high plant regeneration frequency, high green plant production and green plant percentage, could be selected from the evaluated populations (Table 1). For example, anther donor 47 from population 3 had 71% embryogenic anthers and produced 646 ELS per 100 anthers, from which 1002 plants including 858 green plants (86%) per 100 AC could be regenerated.

Influence of paircross parents' androgenic capacity

Different combinations of pair cross parents influenced the performances of the populations for the evaluated traits. For the percentage of embryogenic anthers for example, progeny from a cross between non-androgenic P133 and androgenic P102 or P169 performed significantly better ($P \leq 0.05$) compared to progeny from a cross between P133 and androgenic P2 (Fig. 4a-II). Similarly, anthers of

Table 1 Overview of the 10 anther donor plants with the highest androgenic capacity in terms of percentage of embryogenic anthers, embryo production, total plant production and the proportion of green plants

Genotype	Embryogenic anthers (%)	ELS per 100 AC	Plants per 100 AC	Plants per 100 EC	Green plants per 100 AC (%)	Green plants per 100 EC (%)
Population 2						
25	81	665	594	89	594 (100)	89 (100)
45	57	377	588	156	588 (100)	156 (100)
Population 3						
12	60	406	333	82	333 (100)	82 (100)
47	71	646	1002	155	858 (86)	133 (86)
Population 4						
25	70	607	1050	173	821 (78)	135 (78)
Population 6						
27	54	234	412	176	375 (91)	160 (91)
44	65	281	284	101	245 (86)	87 (86)
47	86	457	1810	396	1530 (85)	335 (85)
Population 10						
30	52	243	506	208	376 (74)	155 (74)
44	64	328	578	176	567 (98)	173 (98)

AC anthers cultured, ELS embryo-like structures, EC ELS cultured

Fig. 4 Box plots with attached density plots of the percentage of embryogenic anthers. **a** Grouped by common non-androgenic paircross parent (P10, P133, P144, P175 and P48); **b** grouped by common androgenic paircross parent (P169, P102 and P2). Combination P133 and P2 performed significantly worse ($P \leq 0.05$) than the other

combinations in section **a-II**. The cross between P169 and P133 performed significantly better ($P \leq 0.01$) than the cross P169 and P10 (**b-I**). Combination P2 and P175 performed significantly worse than all other combinations ($P \leq 0.001$; **b-III**)

genotypes with androgenic P169 as one of their parents were significantly more embryogenic ($P \leq 0.01$) if the non-androgenic parent was P133 instead of P10 (Fig. 4b-I).

The same effect was observed for plants and albino plants per 100 AC ($P \leq 0.05$; Supplementary Table S2). Progeny of the cross between androgenic P2 and non-androgenic

P175 had a significantly lower performance for percentage of embryogenic anthers ($P \leq 0.001$), ELS per 100 AC ($P \leq 0.01$), plants per 100 AC ($P \leq 0.01$) and green plants per 100 AC ($P \leq 0.05$) than progeny of the cross between androgenic P2 and most other non-androgenic parents (Fig. 4b-III; Supplementary Table S1).

Correlations between traits and years

Ranked correlations (Spearman's ρ) were calculated between the evaluated traits, using observations from all populations evaluated in 2015, but excluding genotypes that produced <20 plants per AC ($n = 102$) in order to detect inter-trait relationships relevant in more androgenic material (Table 2). Many positive and highly significant ($P \leq 0.001$) correlations were found, such as $\rho = 0.80$ between plants per 100 AC and green plants per 100 AC. Embryo production had a high positive correlation with plant production ($\rho = 0.83$; $P \leq 0.001$) but was not significantly correlated with plant regeneration. Green plant production had a relatively low, positive correlation with albino production ($\rho = 0.20$; $P \leq 0.05$) and was not significantly correlated with albino regeneration. Green plant regeneration was not significantly correlated with albino plant production and regeneration.

Populations 1, 4, 6 and 7, selected because of their widely segregating and high responses to in vitro AC, were evaluated again in 2016. Due to flower availability, the number of genotypes evaluated in both 2015 and 2016 ($n = 78$) is rather low in all but population 1 ($n = 45$). A clear effect of the year was observed and the anther donors performed significantly better in 2015 for almost all traits (Fig. 5). Ranked positive correlations between the years ranged from $\rho = 0.45$ for plants or green plants per 100 EC ($P \leq 0.001$) to $\rho = 0.54$ for the two embryo related traits ($P \leq 0.001$).

Green regenerants

Loss of green plantlets during and after transplantation to the greenhouse was negligible and 1111 green putative DHs were raised. The selected markers were informative for 529 plants, 99.6% of which were homozygous at loci where the anther donor was heterozygous, confirming their microspore origin. The ploidy levels of all 1111 green regenerants were analysed with flow cytometry and 472 haploid (n), 572 diploid ($2n$), 48 tetraploid ($4n$), and even a few triploid ($3n$), heptaploid ($7n$), and mixoploid (e.g. n and $2n$) plants were found. In the summer of 2016, 539 diploid and 40 tetraploid green regenerants were transplanted into the field, where the conditions were significantly hotter and dryer than most years. Both vigorously growing and extremely weak phenotypes were observed. On a scale from 0 to 9 for overall vigour, 3.7% of the diploid plants scored a 7 or higher, while all of the tetraploid plants scored a 6 or lower. Spikes were produced by 69 diploid and 1 tetraploid plants, out of which 11 diploid plants set seed.

Discussion

Androgenic capacity

In this study, we applied an effective in vitro AC protocol to a large number of perennial ryegrass genotypes derived from crosses between distinct androgenic and non-androgenic plants, and generated thousands of microspore-derived embryos and green DH plants. Interestingly, even though it is difficult to compare between tissue culture studies (Oleszczuk et al. 2014; Seldimirova and Kruglova 2015), quite a few genotypes outperformed the most androgenic plants reported to date, except for our earlier pilot study (Begheyn et al. 2016b). For example, ten anther

Table 2 Ranked correlations (Spearman's ρ) between traits evaluated in 2015, calculated over all populations, excluding genotypes that produced <20 plants per 100 anthers cultured ($n = 102$)

Trait	EA (%)	ELS/100AC	P/100AC	P/100EC	GP/100AC	GP/100EC	A/100AC
ELS/100AC	0.81***						
P/100AC	0.68***	0.83***					
P/100EC	ns	ns	0.66***				
GP/100AC	0.55***	0.60***	0.80***	0.65***			
GP/100EC	0.26**	0.25*	0.56***	0.72***	0.89***		
A/100AC	0.38***	0.58***	0.66***	0.37***	0.20*	ns	
A/100EC	ns	ns	0.21*	0.48***	ns	ns	0.65***
%GP	0.27**	0.23*	0.41***	0.44***	0.83***	0.91***	-0.29**

Correlations between all traits and the percentage of albinos are exactly the same as for the percentage of green plants, only negative and positive are reversed

A albinos, AC anthers cultured, EA embryogenic anthers, EC ELS cultured, ELS embryo-like structures, GP green plants, P plants, ns not significant

*** $P \leq 0.001$; ** $P \leq 0.01$; * $P \leq 0.05$

Fig. 5 Box plots of the responses of populations 1, 4, 6 and 7 to anther culture in both 2015 (*left*) and 2016 (*right*). Spearman's ρ for the overall correlation between years per trait is given within the plots. Significance levels of the differences between the years for each trait and population are given above the plots. **a** Percentage of embryogenic anthers; **b** number of embryo-like structures (ELS) per 100

anthers cultured (AC); **c** number of plants regenerated per 100 AC; **d** number of plants regenerated per 100 ELS cultured (EC); **e** number of green plants (GP) regenerated per 100 AC; **f** number of GP regenerated per 100 EC. For graphical reasons, observation 1530 from population 6 (2015) has been omitted from graph **e**. *** $P \leq 0.001$; ** $P \leq 0.01$; * $P \leq 0.05$; *ns* not significant

donors produced more than 354 green plants per 100 AC, which is three times the highest number reported before (Opsahl-Ferstad et al. 1994b). One highly androgenic genotype from population 6 generated over 1500 green plants per 100 AC, which is comparable to yields of the responsive model barley cultivar 'Igri' (Jacquard et al. 2006) but far better than 'Svilena', the model used in wheat (Lantos et al. 2013). These high yields are probably due to the superior genetics of the studied plant material rather than the relatively minor differences between our method and previous practices (Olesen et al. 1988; Opsahl-Ferstad et al. 1994a, b). Indeed, an unprecedentedly large number of beneficial alleles should be present within the paircross parents, since they originate from a recurrent selection programme to improve androgenicity (Niels Roulund, personal communication; Andersen et al. 1997). Further increases in production efficiency and yield may be achieved by utilizing

recent novel understanding of microspore embryogenesis processes (Žur et al. 2014; Fábíán et al. 2015; Sinha and Eudes 2015) and progress in optimization of (pre-)culture conditions in other cereal species such as barley, wheat or triticale (Castillo et al. 2014; Würschum et al. 2015; Echávarri and Cistué 2016).

Genotypes with a uniquely high androgenic capacity could be selected in the present study (Table 1) and can now be used for the introgression of this trait into recalcitrant germplasm (Halberg et al. 1990; Madsen et al. 1995). In fact, population 11, derived from the cross between androgenic P2 and non-androgenic P175, may be considered as a test case of this 'inducer' approach (Halberg et al. 1990). Even though one of its parents is incapable of microspore embryogenesis or plant regeneration, population 11 included genotypes regenerating as many as 64 green plants per 100 AC. Recalcitrant genotypes can

thus be ‘induced’ to exhibit acceptable productivity levels (Murigneux et al. 1994; Madsen et al. 1995).

Genetic control of androgenic capacity

The evaluated populations showed a continuous distribution for each component trait of androgenic capacity, which confirms their previously reported quantitative polygenic control (Halberg et al. 1990; Opsahl-Ferstad et al. 1994b; Madsen et al. 1995). Additionally, variation in the inheritance patterns between the populations indicates the presence of different numbers of distinct alleles in their pair-cross parents (Madsen et al. 1995; Torp et al. 2001; Nielsen et al. 2015). For example, non-androgenic parent P133 may harbour superior alleles for microspore embryogenesis and plant regeneration compared to P10.

Because all non-androgenic parents in this investigation (except those of populations 10 and 11) are capable of microspore embryogenesis and albino plant regeneration, the remarkably high levels of embryo and plant production observed in their offspring could be due to additive allelic effects or the presence of similar or identical alleles in both parents (Fig. 2). Alleles from androgenic parent P2 must be responsible for the plant regeneration, and possibly also the green plant regeneration, observed in population 10, since its non-androgenic parent (P144) lacks the ability to regenerate plants. This would suggest a certain level of dominance of the genetic control of these two traits (Opsahl-Ferstad et al. 1994b). Although populations 10 and 11 share androgenic parent P2, the non-androgenic parent of the latter (P175) is unable to produce embryos. This resulted in a significantly lower embryo and therefore plant production in population 11 compared to population 10.

Embryo and plant production capacity were highly correlated ($\rho=0.83$; $P\leq 0.001$), suggesting their control by related, linked or even identical genes. This scenario was also proposed in maize, where one QTL was found to be correlated with both traits (Murigneux et al. 1994). Indeed, all traits expressed as production per 100 AC were highly and significantly correlated, which is to be expected since the reverse would be biologically impossible. Drawing conclusions on genetic control from these relationships is, therefore, likely to be of limited value. More informative is, for example, the insignificant correlation observed between embryo production and plant regeneration, which supports the hypothesized distinct genetic control of the two traits in perennial ryegrass (Stanis and Butenko 1984; Olesen et al. 1988; Boppenmeier et al. 1989). In both wheat and barley, no genetic correlation between these two traits was found either (Tuvešson et al. 1989; Larsen et al. 1991).

Conversely, the high correlation between plant and green plant regeneration ($\rho=0.72$; $P\leq 0.001$), again supported by findings in wheat (Tuvešson et al. 1989), is

unlikely to be due to similar genetic control mechanisms, since many of the non-androgenic parents are capable of plant but not green plant production. Additionally, the absence of a correlation between QTL explaining variation in green plant production with embryo formation or plant regeneration was reported in wheat (Torp et al. 2001; Nielsen et al. 2015). Several other possibilities may be considered instead; for example, dominant control of green plant production by several genes, alleles for which are contributed solely by the androgenic parents (Tuvešson et al. 1989; Opsahl-Ferstad et al. 1994b), or complementation of recessive beneficial alleles present in a heterozygous state within the non-androgenic parents (Madsen et al. 1995). Alternatively, environmental conditions before or during AC could have been more conducive to green plant production than when the androgenic capacity of the pair-cross parents was examined. External influences at that time may have caused potential green regenerants to be afflicted by albinism, thus masking the presence of the capacity for green plant production of those genotypes now classified as non-androgenic (see also below) (Opsahl-Ferstad et al. 1994b).

Albinism

The evaluated populations and genotypes differed in the number and percentage of regenerated albinos. Similar to reports in triticale, a low or insignificant correlation was found between green and albino plant production as well as regeneration (González et al. 2005; Krzewska et al. 2015). Opsahl-Ferstad et al. (1994b) found an insignificant correlation of $r=0.29$, similar to the $\rho=0.20$ ($P\leq 0.05$) found here, and hypothesized that different genes must be responsible for green and albino plant production. Also in accordance with their results, a highly negative correlation ($\rho=-0.83$; $P\leq 0.001$) between the number of green plants produced and the percentage of albinos was found, which is expected in the presence of both green and albino plant production promoting alleles in a population (Opsahl-Ferstad et al. 1994b). Additionally, the low negative correlation ($\rho=-0.29$; $P\leq 0.01$) between the number of albino plants produced and green plant percentage, suggests that the environment is of greater influence on albino production than genetics (Kumari et al. 2009; Makowska and Oleszczuk 2014), even though QTL for albinism have been reported in several cereal species (He et al. 1998; Chen et al. 2007; Krzewska et al. 2015). The presence of alleles required for green plant production can thus be ‘phenotypically invisible’, if potential green regenerants suffer from environmentally induced albinism.

Robustness of androgenic capacity

A significant effect of the year was observed in the response to AC and a better overall performance of the genotypes was recorded in 2015, possibly due to late and erratic flowering caused by incomplete vernalization in 2016 (Fig. 5). Influence of the growing conditions of the anther donor has been well documented in perennial ryegrass, and responses may be better at the start of the growing season (Olesen et al. 1988; Boppenmeier et al. 1989; Bante et al. 1990). Similarly, anther response dropped around 50% when barley spikes were harvested in the second half of the year (Jacquard et al. 2006), and seasonal variations in AC response levels of the same genotype was reported in oat (Kiviharju et al. 2017). Higher correlations than the observed $\rho=0.45\text{--}0.54$ ($P\leq 0.001$) would probably have been found between the years, if 2016 had contributed less zeros to the dataset. Nevertheless, genotypes with a high embryo and/or plant production in 2015 were frequently highly androgenic in 2016 as well. One genotype from population 4 produced the most total plants and green plants in both years for example. Indeed, the three best green plant producers within population 1 in 2016 were in the top five in 2015.

To investigate the stability of androgenic responses over time and environments, several highly androgenic genotypes were artificially vernalized and induced to flower in a climate chamber during the winter of 2016. Many embryos and green plants were produced under such conditions as well (data not shown). Based on these findings, it seems that highly androgenic genotypes do exhibit a robust DH production and, as has been reported elsewhere, the heritability of this ability is relatively high (Lazar et al. 1984; Opsahl-Ferstad et al. 1994b; Moieni et al. 1997).

Characteristics of the green regenerants

Andersen et al. (1997) reported that 99.7% of the 913 green regenerants they screened were homozygous, a nearly identical finding to the 99.6% homozygosity observed in this study. Perennial ryegrass AC does not seem to be sensitive to undesirable regeneration of heterozygous plantlets from sporophytic tissue or unreduced gametes. The observed spontaneous chromosome doubling rate of 52% of this study is at the lower end of the 50–80% range reported in literature (Olesen et al. 1988; Bante et al. 1990; Halberg et al. 1990; Begheyn et al. 2016b). Besides spontaneous polyploidization, chromosome doubling may also be induced by in vitro or in vivo application of chromosome doubling agents such as colchicine (Melchinger et al. 2016; Ślusarkiewicz-Jarzina et al. 2017). In contrast to triticale (Lantos et al. 2014) and wheat (Rubtsova et al. 2013) however, this is not considered necessary in perennial ryegrass

due to a high level of natural chromosome doubling (Andersen et al. 1997).

Our field evaluation of almost 600 regenerated DHs confirmed that, even though most were weak and showed low fertility, it was possible to select vigorous and fertile genotypes, similar to findings reported by Bante et al. (1990), Opsahl-Ferstad (1993) and Andersen et al. (1997). Perennial ryegrass DHs perform similar to those produced in rye, another obligate outcrossing member of the grass family in which AC is being studied. One investigation, performed on fewer genotypes than used here, found that only 10–36% of rye DHs were suitable for research or breeding purposes due to low vigour, fertility, survival and abnormal growth (Tenhola-Roininen et al. 2006). On the other hand, the maintenance and multiplication of DHs of tall fescue (*Lolium arundinaceum* (Schreb.) Darbysh. [syn. *Festuca arundinacea* Schreb.]) through seed obtained by self-fertilization was not found to pose any problems, even though this species is known to be self-incompatible (Kindiger 2016). More investigations into the vigour and fertility of perennial ryegrass DHs are needed in order to better characterize to what extent inbreeding depression affects their agronomic performance.

Concluding remarks

Here, we have demonstrated the effectivity of the present in vitro AC method for DH production in perennial ryegrass, as well as the value of the used plant material as a resource for future inquiries into the genetic control of androgenic capacity. The variation in embryo production, plant regeneration and green plant production observed between and within the populations was large, and its pattern indicates the presence of different genes and distinct alleles involved in the control of androgenic capacity. Through approaches like GWAS or QTL mapping, genomic regions involved in the control of microspore embryogenesis, plant regeneration, green and albino plant production may now be identified within this germplasm (Wędzony et al. 2015). Apart from their academic interest, these types of studies facilitate the development of molecular markers for androgenic ability, which, in concert with highly responsive ‘inducer’ genotypes such as the ones selected here, could rapidly and dramatically increase the perennial ryegrass gene pool from which DHs can efficiently be produced. This expansion of the applicability of DH techniques has the potential to accelerate the progress of basic and applied research as well as breeding programs in the forage grasses.

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Author contributions BS, NR and KV conceived the idea, designed the experiments and edited the manuscript. NR and KV developed the methodology and the in vitro protocol. RB conducted the experiments, analysed the data and wrote the manuscript. DK facilitated and helped conduct the flow cytometry experiments and edited the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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