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Towards efficient doubled haploid production in perennial ryegrass (*Lolium perenne* L.)

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Summary

The ability to produce doubled haploid plants has found broad application in fundamental and applied research as well as in practical plant breeding. In maize (*Zea mays* L.) and barley (*Hordeum vulgare* L.), for instance, routine, large-scale doubled haploid production in a single generation has replaced the 5-7 cycles of repeated self-fertilization formerly required to obtain sufficiently homozygous material. Doubled haploid lines have been invaluable for robust, multi-year phenotyping in quantitative trait loci mapping studies and their complete homozygosity has simplified genome sequence assembly efforts. Furthermore, doubled haploids are directly released as stable, homogeneous cultivars or used as parents of F1 hybrid seed to exploit the phenomenon of heterosis. At this time, however, neither homozygous line nor doubled haploid production can be efficiently applied in the important forage crop perennial ryegrass (*Lolium perenne* L.). An effective self-incompatibility system as well as a marked sensitivity to inbreeding depression hamper iterations of self-fertilization, while low and highly genotype-specific responses render the available in vitro doubled haploid induction methods unproductive. Major benefits associated with easy access to homozygous germplasm, particularly the straightforward production of high yielding hybrid varieties, are therefore unavailable to those working with this economically significant species.

This thesis documents the initiation of a modern reboot of the development of an efficient doubled haploid production system for perennial ryegrass. The main goal was to obtain a level of understanding of the genetic factors, as well as their genomic locations, governing the response to in vitro doubled haploid induction, that would enable rapid and effective introduction of high levels of androgenic ability into recalcitrant germplasm. Four distinct phases of the research are reported in chapters 2 through 5, which are summarised below.

First of all, a comprehensive overview of the ways in which haploid and doubled haploid techniques may be exploited to reduce the time, space and investment associated with perennial ryegrass research and breeding, is given in **chapter 2**. The potential of doubled haploid induction to 1) purge deleterious alleles from germplasm intended for breeding, 2) develop mapping populations for genetic and genomic studies, 3) simplify haplotype mapping, 4) fix transgenes and mutations for functional gene validation and molecular breeding, and 5) hybrid cultivar development is discussed. It is concluded that relatively minor improvements to existing in vitro doubled haploid induction protocols of perennial ryegrass should make some exceedingly useful applications available to the forage grass community.

A pilot study to characterize the responses of putative highly androgenic germplasm to a decades old in vitro anther culture protocol is presented in **chapter 3**. The method proved to be efficient and large numbers of microspore-derived embryo-like structures as well as green and albino plantlets could be recovered. In addition, significant genotype-dependent variation was observed between the individual anther donors, confirming the usefulness of the plant material for further investigations into the androgenic ability of perennial ryegrass.

Chapter 4 describes the results of the subsequent large, two year screen of the response to the in vitro anther culture protocol of nine distinct, bi-parental populations, obtained by crossing two genotypes with contrasting androgenic capacities. The variation in embryo production, plant regeneration and green plant production observed between and within the populations was large, and its pattern indicated the presence of different genes and alleles involved in the control of the component traits of androgenic ability. An insignificant association of embryo production with plant regeneration, as well as a low correlation between green and albino plant yield, suggested that distinct genes influence these traits. Furthermore, the environment was found to affect the incidence of

albinism to a larger degree than genetic components. Lastly, it was concluded that the evaluated populations could provide rare, beneficial alleles for the introgression of high levels of androgenic capacity into recalcitrant material.

Finally, **chapter 5** addresses the identification of genetic loci associated with androgenic ability, which were determined via a powerful genotyping-by-sequencing-based genome-wide association study using the plant material and phenotypic data of the previous chapter. Between 1 and 10 quantitative trait loci were identified for anther response, embryo and total plant production, green and albino plant production and regeneration. Interestingly, a locus with a major effect on green plant regeneration was identified on perennial ryegrass linkage group 5 which may prove to be orthologous to loci detected at a similar genomic location in four related Poaceae species. In addition, two intriguing candidate genes, encoding chromatin binding domains of the developmental phase transition regulator, Polycomb Repressive Complex 2, were identified and merit further investigation. Ultimately, these results currently enable the development of molecular markers to rapidly introgress androgenic capacity into recalcitrant perennial ryegrass germplasm.

It is hard to overstate the importance of doubled haploid techniques to advance research and increase the speed of genetic gain of breeding programmes. Here, I have demonstrated the effectivity of an in vitro anther culture method for doubled haploid production in perennial ryegrass, a forage species of global importance. In addition, plant material carrying valuable alleles as well as the molecular tools to identify them are now available, so that efficient doubled haploid production may soon be added to the arsenal of research and breeding tools of the perennial ryegrass community. My work will significantly accelerate forage grass breeding and constitute a key step towards the realization of a grass hybrid breeding programme. Finally, the results obtained here may prove to be of significant benefit to research and breeding efforts in related Poaceae species as well.

Résumé

La capacité de produire des plantes haplodiploïdes a trouvé une large application dans la recherche fondamentale et appliquée ainsi que dans l'amélioration des plantes. Pour le maïs (*Zea mays* L.) et l'orge (*Hordeum vulgare* L.) par exemple, la production d'haplo-diploïdes à grande échelle, en une seule génération, a remplacé les 5-7 cycles d'autofécondation préalablement nécessaires pour obtenir un matériel suffisamment homozygote. Les lignées haploïdes doublées ont été inestimables pour un phénotypage robuste et pluriannuel dans des études de cartographie des loci quantitatifs et leur homozygotie absolue a simplifié les efforts d'assemblage de séquences génomiques. En outre, les haploïdes doublés sont directement commercialisés sous la forme de cultivars stables et homogènes ou utilisés pour exploiter le phénomène de l'hétérosis en tant que parents de semences hybrides F1. En ce moment, cependant, ni la lignée homozygote ni la production haploïde doublée ne peuvent être appliquées efficacement au ray-grass anglais (*Lolium perenne* L.), importante culture fourragère. Un système d'auto-incompatibilité efficace ainsi qu'une sensibilité marquée à la dépression de consanguinité entravent les itérations d'autofécondation, tandis que les réponses faibles et hautement génotypiques rendent improductives les méthodes d'induction d'haploïdes doublés multipliés in vitro. Les principaux avantages associés à un accès facile à un germoplasme homozygote, en particulier la production directe de variétés hybrides à haut rendement, sont donc indisponibles pour ceux qui travaillent avec ces espèces économiquement significatives.

Cette thèse documente un redémarrage moderne du développement d'un système efficace de production de haplodiploïdes pour le ray-grass pérenne. L'objectif principal était d'obtenir un niveau de compréhension des facteurs génétiques, ainsi que de leurs sites génomiques, régissant la réponse à une induction d'haploïdes doublés in vitro, ce qui permettrait une introduction rapide et efficace de niveaux élevés de capacité androgénique dans le germoplasme récalcitrant. Quatre phases distinctes de la recherche sont présentées dans les chapitres 2 à 5, qui sont résumées ci-dessous.

Tout d'abord, un aperçu complet des façons dont les techniques haploïdes et haploïdes doublés peuvent être exploitées pour réduire le temps, l'espace et l'investissement associés à la recherche et à l'amélioration du ray-grass anglais est donnée au **chapitre 2**. L'induction d'haploïdes doublés est discutée quant à son potentiel à 1) purifier les allèles délétères du germoplasme destiné à la sélection, 2) développer des populations cartographiques pour les études génétiques et génomiques, 3) simplifier la cartographie des haplotypes, 4) fixer les transgènes et les mutations pour la validation fonctionnelle des gènes et la sélection moléculaire, et 5) favoriser le développement de cultivars hybrides. On conclut que des améliorations relativement mineures aux protocoles d'induction d'haploïdes doublés in vitro existants pour le ray-grass anglais devraient rendre disponibles certaines applications extrêmement utiles à la communauté d'amélioration des graminées fourragères.

Une étude pilote pour caractériser les réponses d'un germoplasme présumé hautement androgène à un protocole de culture d'anthères in vitro âgé de décennies est présentée au **chapitre 3**. La méthode s'est révélée efficace et un grand nombre de structures embryonnaires dérivées de microspores a pu être récupéré, ainsi que de nombreuses plantules vertes et albinos. En outre, une variation significative dépendante du génotype a été observée entre les individus donneurs d'anthères, confirmant l'utilité de ce matériel végétal pour d'autres recherches sur la capacité androgénique du ray-grass anglais.

Le **chapitre 4** décrit les résultats d'un large screening de deux ans de la réponse d'anthères au protocole de culture in vitro, portant sur neuf populations biparentales distinctes, obtenues en croisant deux génotypes avec des capacités androgéniques contrastées. La variation observée intra et inter-populations en termes de production d'embryons, de régénération de plantules et de production de plantules vertes était importante, et sa distribution indiquait la présence de différents gènes et allèles impliqués dans le contrôle des caractères liés à la capacité androgénique. Une association non-

significative entre la production d'embryons et la régénération des plantes, ainsi qu'une faible corrélation entre le production des plantes vertes et de plantes albinos, ont suggéré que des gènes distincts influencent ces caractères. De plus, l'environnement est apparu comme ayant une influence sur l'incidence de l'albinisme supérieure à celle des composants génétiques. Enfin, il a été conclu que les populations évaluées pouvaient fournir des allèles bénéfiques rares pour l'introgression de niveaux élevés de capacité androgénique dans du matériel végétal récalcitrant.

Enfin, le **chapitre 5** traite de l'identification des loci génétiques associés à la capacité androgénique, qui ont été déterminés par une étude d'association pangénomique (genome-wide association study) basée sur un puissant génotypage par séquençage, utilisant le matériel végétal et les données phénotypiques du chapitre précédent. Entre 1 et 10 loci de caractères quantitatifs (quantitative trait loci) ont été identifiés pour la réponse des anthères, la production d'embryons et la production végétale totale, la production et la régénération des plantes vertes et albinos. Fait intéressant, un locus ayant un effet majeur sur la régénération de plantes vertes a été identifié sur le groupe de liaison 5 de ray-grass anglais, qui pourrait s'avérer orthologue aux loci détectés à un emplacement génomique similaire dans quatre autres espèces de poacées. En outre, deux gènes candidats intrigants, codant pour les domaines de liaison à la chromatine du régulateur de transition de phase de développement, Polycomb Repressive Complex 2, ont été identifiés et méritent d'être approfondis. Enfin, ces résultats permettent dès a présent le développement de marqueurs moléculaires pour introduire rapidement une capacité androgénique dans un germoplasme de ray-grass pérenne récalcitrant.

Il est difficile d'exagérer l'importance des techniques d'haplodiploïdisation pour faire progresser la recherche et augmenter la vitesse de gain génétique des programmes de sélection. Ici, j'ai démontré l'efficacité d'une méthode de culture d'anthères in vitro pour la production d'haplodiploïdes dans le ray-grass anglais, une espèce fourragère d'importance mondiale. En outre, le matériel végétal portant des allèles précieux ainsi que les outils moléculaires pour les identifier sont maintenant disponibles, de sorte qu'une production efficace d'haplodiploïdes peut bientôt être ajoutée à l'arsenal des outils de recherche et de sélection de la communauté du ray-grass anglais. Mon travail accélérera considérablement l'amélioration des graminées fourragères et constituera une étape clé vers la réalisation d'un programme de sélection de ray-grass hybrides. Enfin, les résultats obtenus ici peuvent s'avérer très utiles pour la recherche et l'amélioration des espèces de poacées.

1. Introduction

1.1 The significance of perennial ryegrass

Perennial ryegrass is a naturally diploid member of the grass family (Poaceae) and native to temperate Asia, Europe and North Africa. Its close relatives include major crop species such as barley (*Hordeum vulgare* L.), rice (*Oryza sativa* L.), maize (*Zea mays* L.), sorghum (*Sorghum bicolor* L.) and wheat (*Triticum aestivum* L.), as well as the model grass *Brachypodium distachyon*. Perennial ryegrass is used to mitigate soil erosion (de Baets *et al.* 2011), exploited for phytoremediation of abandoned industrial sites (Komárek *et al.* 2013) and even classified as a problematic weed with high levels of herbicide resistance in some parts of the world (Hussain and Reigosa 2017). Turf types are sown in stadiums and golf courses, parks and domestic lawns, where their aesthetically pleasing greenness as well as the soft surface they provide, enhance our experience of sport and recreation. In agriculture, perennial ryegrass forage varieties are used to establish temporary pastures used for grazing or feed production (Reheul *et al.* 2010). Farmers of the world's temperate zones appreciate its excellent productivity, good persistence and high nutritional value for ruminant livestock. When properly managed, grasslands provide a cheap source of feed for meat and dairy cattle (Lawrence *et al.* 2017). Optimum yield and feeding value is achieved when the swards are cut just prior to the formation of generative stems (Ullmann *et al.* 2017), which has resulted in a classification of commercial cultivars by heading date. In contrast to natural, permanent grasslands, temporary pastures have to be re-sown regularly to ensure their productivity (Reheul *et al.* 2007). A pasture renewal rate of 6-8% per year, for example, is the New Zealand average (McNally *et al.* 2017). At such times, farmers turn to grass breeding companies to purchase novel varieties, bred for ever better on-farm performances as well as enhanced feed quality.

1.2 Perennial ryegrass breeding

Organized breeding of perennial ryegrass is a relatively recent activity and was first established in post-World War I Britain to meet demands for higher pasture productivity. The decades following the first commercial cultivar releases in the 1930s, saw public and private institutions in Europe, the United States, Australia and New Zealand initiate programmes of their own (Humphreys 2005). In the 1970s, breeding practices shifted from polycrosses of landraces and ecotypes to recurrent selection within populations of heterozygous individuals. Synthetic, genetically heterogeneous perennial ryegrass cultivars were then produced by intercrossing distinct, improved parent populations for several generations (Brummer 1999). Around the same time, the first tetraploid cultivars were released, which were artificially produced using the chromosome doubling agent colchicine (den Nijs and Stephenson 1988). In general, tetraploids are more palatable than diploids, but they yield a less dense sward and exhibit lower grazing tolerance as well (Smith *et al.* 2001). During the 1980s and 1990s, the popularity of forage ryegrasses rose significantly, due, in part, to their excellent performance under high nitrogen regimes. Following technological advances, molecular marker-assisted selection (MAS) and, a decade later, genomic selection (GS) were added to the breeders' tool box (Kölliker *et al.* 2005; Hayes *et al.* 2013). Perennial ryegrass now dominates the forage grass seed market in places such as Europe and Australia (Sampoux *et al.* 2011).

Perennial ryegrass cultivar development is a relatively lengthy process, taking between 10-14 years, due to the multi-year observations required for effective assessment of yield and quality-related traits (Hayes *et al.* 2013). Biomass yield, persistency and disease resistance have long been the main criteria for variety cultivation and use (VCU) trials (Wilkins 1991). Later, palatability, digestibility and feed value were added as important breeding and variety evaluation traits. Genetic improvement of seed yields, which are currently a rather low 1200 – 2500 kg ha^{-1}, is a relatively recent breeding objective

(Conaghan and Casler 2011). Variations in seed yield directly affect the profits of the breeding companies themselves. Additionally, with improved understanding of the symbiosis between perennial ryegrass and endophytic fungi, both breeding and agricultural practices aimed at exploiting its potential benefits have become of interest (Johnson *et al.* 2013; Hume and Sewell 2014). Overall, genetic gains achieved by perennial ryegrass breeders have improved animal productivity (Hendriks *et al.* 2017). The economic merit of varieties can even be estimated via ranked indices, so that farmers in New Zealand (Chapman *et al.* 2017) and Ireland (O'Donovan *et al.* 2017), for example, may select the most profitable cultivar given their particular agricultural circumstances.

While population-based selection has definitely been successful in perennial ryegrass breeding, the relatively inefficient exploitation of heterosis inherent to this approach has been commented on by many authors (Brummer 1999; Kölliker *et al.* 2005; Bolaric *et al.* 2005; Posselt 2010; Arias Aguirre *et al.* 2011; Connor *et al.* 2015; Pembleton *et al.* 2015). Heterosis, sometimes referred to as hybrid vigour, is the greater size, productivity or tolerance to adverse conditions that may be observed in cross-bred individuals compared to their (more) inbred counterparts or parents (East 1936; Schnable and Springer 2013). This phenomenon is effectively exploited by means of F1 hybrid breeding, in crops such as maize (Geiger and Gordillo 2009), rapeseed (Yamagishi and Bhat 2014), sunflower (Schnabel *et al.* 2008) and rice (Cheng *et al.* 2007). Several obstacles need to be overcome to realize hybrid perennial ryegrass breeding. Examples of these are the absence of an effective pollination control mechanism, well-defined and characterized heterotic groups to direct the compatibility of parental lines and, most relevant here, the challenges of inbreeding an obligate outcrossing species (Yang *et al.* 2008). In this last respect, the production of DHs is most likely to be the most efficient way to produce homozygous lines of perennial ryegrass.

The implementation of novel breeding technologies has been recognized as one of the most important ways to accelerate crop improvements, so that the urgent, global need for increased plant productivity may be realized and the demands of a rapidly growing population met (Tester and Langridge 2010). In perennial ryegrass breeding programmes, DH production is an example of such a novel technique of great potential benefit.

1.3 Doubled haploid production

The full significance of the ability to efficiently produce DHs as well as their potential applications for perennial ryegrass research and breeding are discussed in detail in **chapter 2**. Apart from emphasising that the availability of DH technologies does not solely benefit breeding but many research endeavours, such as genome sequencing, linkage mapping studies and mutation experiments, too, a brief introduction to DH production will suffice here.

A distinction between in vivo and in vitro DH induction is often made. The former includes haploid induction via wide hybridization followed by uniparental genome elimination, for example via crosses between barley and *H. bulbosum* (the 'Bulbosum method') (Kasha and Kao 1970), wheat and maize (Niu *et al.* 2014), oat (*Avena sativa* L.) and maize (Nowakowska *et al.* 2015), or maize and *Imperata cylindrica* (Chaudhary *et al.* 2013). A second in vivo approach is the use of intraspecific crosses with haploid inducer genotypes, which is a widespread practice in maize hybrid breeding (Prigge *et al.* 2012). To complete the production of fertile DH lines, embryo rescue and/or artificial chromosome doubling are usually required after in vivo haploid induction. The production of DHs via any in vitro method is based on the regeneration of plants from cultured, haploid cells and may include artificial chromosome doubling as well (Seguí-Simarro 2010). Both female and male gametophytes are used as starting material in this approach, although gynogenesis is far more laborious and thus only applied if no other methods are available. In onion (*Allium cepa* L.), for instance, ovary culture is the only viable method of DH production (Fayos *et al.* 2015). Anthers and pollen are usually available in abundance

and easily harvested, which accounts for the popularity of in vitro androgenesis. Protocols based on anther culture (AC) or isolated microspore culture (IMC) have been developed for hundreds of species (Maluszynski *et al.* 2003; Touraev *et al.* 2009). Nevertheless, large-scale, routine and commercial application of AC and IMC is currently restricted to barley, tobacco (*Nicotiana* spp.) and rapeseed (*Brassica napus* L.), due to the generally low and genotype-specific responses (recalcitrance) of most of the germplasm of many commercially significant species (Dwivedi *et al.* 2015). An additional challenge, particular to the grass family, is an often problematically high incidence of albinism amongst the regenerants (Kumari *et al.* 2009). Even so, significant progress in wheat, rye (*Secale cereale* L.), oat, triticale (× *Triticosecale*), potato (*Solanum tuberosum* L.) and cabbage (*B. oleracea* L.) DH production has enabled their more frequent application in recent years.

Perennial ryegrass AC protocols have been under development since the early 1980s (Stanis and Butenko 1984) and have culminated in a method that is highly efficient in amenable germplasm (Andersen *et al.* 1997). Unfortunately, such germplasm is rare, so that widespread application of DH production in perennial ryegrass research or breeding will require genetic and/or methodological improvements (Hussain *et al.* 2006). To fully appreciate the implications of any modifications to either of these aspects, it is necessary to first examine some of the characteristics of the phenomenon responsible for in vitro androgenesis: microspore embryogenesis (ME).

1.4 Microspore embryogenesis

1.4.1 Induction of microspore embryogenesis

Fertilization induces the acquisition of cellular embryogenic competence during sexual hybridization, whereas, in immature microspores, a stress treatment is required to achieve a similar cell fate switch (Hand *et al.* 2016). The primary response of a microspore population is cell cycle arrest, followed by varying frequencies of either immediate or delayed cell death. Any surviving, successfully induced microspores may, if cultured under beneficial in vitro conditions, either develop into callus-like structures, which may regenerate into plants via organogenesis, or undergo ME proper (Seguí-Simarro and Nuez 2008). To achieve successful microspore induction, a species-specific optimum stress type, timing and duration has to be applied (Shariatpanahi *et al.* 2006). Carbohydrate starvation and heat stress are effective in wheat, for example, while a cold treatment proved effective in perennial ryegrass (Opsahl-Ferstad *et al.* 1994a). Callus development, which is considered to be a less complex and demanding process than ME, may be a sign that the stress treatment or culture conditions need further optimization (Seguí-Simarro 2010). In rapeseed, tobacco and barley, up to 70% of microspores can be induced to undergo ME and develop into embryos without callogenesis (Germanà 2011).

Cellular stress responses play a major role in the induction of embryogenesis and an upregulation of stress response-related genes has been reported (Hand *et al.* 2016). A stress intensity needs to be determined at which an optimal balance exists between ME incidence, which is induced by the stress treatment, and cell viability, which is negatively affected by stress. Protocol amendments aimed at reducing early microspore cell death, such as the addition of caspase inhibitors to the induction medium, can be effective (Sinha *et al.* 2016). The stress application also leads to increased abscisic acid (ABA) levels within the microspores, so that cellular stress response pathways, involving heat-shock proteins (HSP), for example, are activated to maintain cell viability (Maraschin *et al.* 2005). Higher endogenous as well as exogenous ABA levels have, in fact, been found to be beneficial to embryo production (Żur *et al.* 2015a). Excessive accumulation of reactive oxygen species (ROS) during a stress treatment, on the other hand, is detrimental to microspore viability. Oxidative stress can be somewhat negated by the addition of ROS scavengers, such as dimethyl sulfoxide (DMSO), although the capacity to maintain endogenous, enzymatic oxidant activity may be more effective (Żur *et al.* 2014; Sinha and Eudes 2015; Echávarri and Cistué 2016). Interestingly, winter cultivars of cereals such as barley and

wheat, often exhibit higher induction efficiencies than spring types, which has been attributed to their inherently higher tolerance to environmental stresses (Lantos *et al.* 2013; Makowska *et al.* 2015). It has also been shown that cultured microspores often produce an excess of ethylene, so that a certain degree of inhibition of its biosynthesis or activity positively influences microspore viability and embryo production. Increasing the CO_2 concentration in the culture vessel, withholding iron during the first few days of IMC or the addition of cobalt or silver thiosulfate (STS) to the culture media, have been beneficial in perennial ryegrass, rapeseed and triticale, respectively (Bante *et al.* 1990; Würschum *et al.* 2015; Leroux *et al.* 2016).

Immature microspores are generally most amenable to ME induction at the uninucleate stage prior to, or directly following, pollen mitosis I (PMI). The assumption is that microspores are fully committed to gametogenesis after the next and final division, PMII. In fact, the immature pollen transcriptome is very similar to that of the sporophyte, while the transcription of genes in mature pollen is highly specialized, which may explain the embryogenic capacity of the former (Whittle *et al.* 2010). Alternatively, the microspores' ability to divide may be lost after PMII (Soriano *et al.* 2013). Starch accumulation within the microspore, a sign of advanced maturation, is associated with non-embryogenic pollen (Daghma *et al.* 2014). During barley ME, starch biosynthesis- and accumulation-related genes are downregulated while starch and sucrose degradation factors are upregulated (Maraschin *et al.* 2006). Indeed, the recalcitrance to androgenesis of the Solanaceae family has been attributed to the much earlier onset of starch accumulation in their microspores (Seguí-Simarro 2016). Conversely, rapeseed microspores accumulate starch at a rather late phase of gametogenesis and can still be successfully induced at the mature, late bicellular stage (Binarova *et al.* 1997).

1.4.2 Microspore embryogenesis

First signs of the successful de-differentiation of microspores into the embryogenic pathway include cell enlargement, vacuolar fragmentation and the appearance of a preprophase band of microtubules, marking the plane of division (Maraschin *et al.* 2005). Autophagy and lysosomal recycling mediate cytoplasmic cleaning and supposedly prepare the cell for developmental reprogramming (Corral-Martínez *et al.* 2013). A 'star-like' morphology may be observed, caused by the relocation of the nucleus from the cellular perimeter to the centre, where it is then surrounded by cytoplasmic strands. Microspores that develop into embryos do not necessarily present these signs, however, while those that do will not always continue to divide (Daghma *et al.* 2012). Interestingly, exogenous factors affecting cytoplasmic restructuring, such as cold stress or n-butanol application, increase ME in species such as maize, wheat and barley (Soriano *et al.* 2008; Broughton 2011; Fábián *et al.* 2015). It has thus been proposed that the disruption of cytoplasmic organization can, in itself, trigger embryogenesis.

A second marker of ME, reported in rapeseed, tobacco, wheat and barley, for example, is the first symmetric division of the microspore. Nevertheless, asymmetric divisions can result in embryo formation as well (Tang *et al.* 2013). It is from this point onward that genome duplication via nuclear fusion can occur, resulting in the regeneration of diploid regenerants (Daghma *et al.* 2014). Species vary in the frequency at which spontaneous chromosome duplication occurs. In winter barley IMC for instance, as many as 80% of the regenerated plants may be diploid (Devaux and K.J. Kasha 2009). To increase the recovery of chromosome doubled regenerants, colchicine is sometimes added to the culture medium of such species as oat, rapeseed and triticale (Zhou *et al.* 2002; Sidhu and Davies 2009; Ślusarkiewicz-Jarzina *et al.* 2017).

Exine rupture at the aperture and subsequent embryo protrusion mark the third phase of ME. Cell polarity, which was conferred by the presence of the aperture up to this point, must be maintained for proper embryo development. Cell death following exine rupture at the opposite side of the aperture, observed in barley ME, for example, is thought to be caused by loss of polarity (Maraschin *et al.* 2005).

The development of the microspore-derived embryo will now closely mimic its zygotic counterpart. Any differences, such as distinct ethylene, auxin and ABA production and accumulation patterns, may be attributed to culture conditions that do not properly compensate for the absence of endosperm (Seguí-Simarro and Nuez 2008). Some authors have proposed that the microspores themselves produce some of the factors not supplied by the missing endosperm. Recalcitrance and low productivity can, for example, be alleviated by using preconditioned media on which dividing microspores have previously been grown. Ovary, or more correctly, pistil pre- or co-culture is used to the same effect in wheat and barley (Broughton 2008; Lippmann *et al.* 2015). Low molecular weight compounds, auxins and arabinogalactan proteins (AGPs) are the suspected 'nurse-factors' by means of which the (increased) embryo production is achieved (Castillo *et al.* 2015). In fact, *EARLY CULTURE ABUNDANT* (*ECA1*), which encodes a candidate nurse-factor and AGP-like secreted protein, was upregulated in dividing barley microspore cultures (Vrinten *et al.* 1999; Pulido *et al.* 2009). At this moment, however, the mechanisms behind nurse-factors' influence remain obscure and attempts to artificially mimic their actions unsuccessful (Żur *et al.* 2015a).

1.4.3 Gene expression during microspore embryogenesis

In general, gene expression as well as transcriptome studies indicate profound physiological, structural and functional cellular changes in induced as well as dividing microspores (Muñoz-Amatriaín *et al.* 2009a; Seifert *et al.* 2016). Since the model species *Arabidopsis* is famously recalcitrant to in vitro androgenesis, investigators have instead focused their efforts on rapeseed, tobacco, barley and, more recently, wheat, where ME efficiencies are high. Due to the diversity of technical methods and species that have been used, however, it is challenging to draw comparisons between experiments. In addition, many of the differentially expressed genes are of unknown function. Furthermore, separating microspores still following the gametophytic pathway from those undergoing embryogenesis, complicates examinations of the very early stages of ME (Soriano *et al.* 2013). The majority of studies have therefore focused on the later phases of ME and many similarities between microspore-derived and zygotic embryos were discovered. These included changes in the expression of the *APETALA 2* (*AP2*) transcription factor *BABY BOOM* (*BBM*), the *LEAFY COTYLEDON* (*LEC*) transcription factors *LEC1*, *LEC2* and *FUS3*, *WUSCHEL* (*WUS*), *AGAMOUS-like 15* (*AGL15*) and *SOMATIC EMBRYOGENESIS RECEPTOR KINASE* (*SERK*) family genes (Hand *et al.* 2016). In rapeseed IMC, for example, overexpression of *LEC1* reduced embryo production but not plant regeneration. Reduced expression of *LEC1* and *FUS3* led to the reduced production of structurally abnormal embryos as well as lower plant regeneration (Elahi *et al.* 2016). Interestingly, *LEC1*, *LEC2* and *FUS3* are all over-expressed in *Arabidopsis* double mutants of two Polycomb Repressive Complex 2 (PRC2) domains, which cannot terminate the embryogenic phase (Bouyer *et al.* 2011). In plants, PRC2 is vital for cellular reprogramming via chromatin remodelling during developmental transitions, from embryo to sporophyte for instance. Chromatin remodelling-related transcripts were found to be differentially expressed between different phases of wheat ME as well (Seifert *et al.* 2016). Members of the small *SERK* gene family encode leucine-rich repeat transmembrane receptor-like kinase (LRR-RLK) proteins and are associated with somatic embryogenesis in, for example, *Arabidopsis*, wheat and rice, as well as in several apomictic species (Hu *et al.* 2005; Singla *et al.* 2008; Podio *et al.* 2014; Ahmadi *et al.* 2016). The expression of *SERK1* and *SERK2* correlated with embryo production as well as plant regeneration during rapeseed IMC (Ahmadi *et al.* 2016). In wheat, on the other hand, *SERK1* expression was highest in freshly harvested microspores and declined in subsequent ME phases (Seifert *et al.* 2016). Further elucidation of the roles of the genes that are differentially expressed during ME will most likely rely on non-destructive live cell-imaging techniques, such as those recently developed for wheat and barley (Daghma *et al.* 2014; Kumlehn 2016).

1.4.4 Albinism and microspore embryogenesis

Albinism involves a complete or partial lack of pigments, which impairs photosynthesis and prohibits an autotrophic lifestyle. In cereal in vitro androgenesis, albinism rates vary from genotype to genotype but can be as high as 100%. In barley, for instance, albinism incidence is higher in spring than in winter cultivars (Makowska and Oleszczuk 2014). Commonly cited causes for albinism include genotype, influence of the in vivo and in vitro environment, incompatibilities between the nuclear and plastid genomes, partial deletions of the plastid genome, defects in the chlorophyll biosynthetic pathways or an impaired photosynthetic system (Kumari *et al.* 2009). Cytological as well as plastid and nuclear genomic studies have been used to elucidate the causal mechanism for the inability of proplastids to transform into chloroplasts. Diverse and sometimes contradictory hypotheses have been formulated, some of which will be discussed below. It is likely that there is no single mechanism responsible for albinism and therefore no single solution to reduce its incidence.

Plastid DNA (ptDNA) degradation has been observed in studies on barley ME and was thus assumed to inhibit plastid differentiation and cause albinism (Caredda *et al.* 2000, 2004). Microspore-derived albinos with intact ptDNA are not uncommon, however, suggesting ptDNA deletions may not be the primary source of albinism but merely a side effect of stress responses prior to or during culture (Touraev *et al.* 2001). Reduced transcription of photosynthetic and ribosomal RNA encoding genes was observed within the plastids as well. A stress induced lack of functional plastid ribosomes was therefore proposed to result in albino regenerants. In fact, Torp and Andersen (2009) hypothesized that genotypes carrying a rare, recessive, lack-of-function mutation on wheat chromosome 2BL do not produce the low molecular weight compounds that ordinarily reduce plastid ribosomal activity during in vitro microspore culture. This reasoning explained the high incidence of albinism in AC of most wheat breeding germplasm as well as the significant increase in green plant production observed in genotypes with the mutation (Torp and Andersen 2009). Indeed, the high rates of albinism in Poaceae species may be explained by the fact that, in contrast to the dicots, the loss of plastid transcription is not lethal in monocots (Kumari *et al.* 2009).

Plastid developmental stage at the time of microspore induction has been implicated in albinism as well. Barley cultivars Igri and Cork exhibit a similar response to in vitro AC, except for their respective green plant regeneration frequencies of 78% and 2% (Jacquard *et al.* 2006). It was observed that Cork microspores at the correct developmental stage for AC contained plastids that were smaller and contained less ptDNA than those in Igri. Since plastid elimination is part of pollen maturation, it was suggested that Cork plastids are further along the gametophytic pathway. Their reprogramming was therefore no longer possible, even though the microspores themselves could still undergo ME. It may thus be possible that the developmental programs of the microspore and its plastids are not synchronized and independent (Makowska and Oleszczuk 2014). Microspores may make the switch to embryogenesis, while their plastids continue on the gametophytic pathway and are eliminated, with albinos as a result. Nevertheless, a major issue with this hypothesis is the fact that, even though plastid maturity is fixed prior to microspore induction treatment, culture conditions can influence the frequencies at which albinos are produced. Optimization of induction stress, medium osmolarity, cytokinin concentration as well as the addition of copper or zinc sulphates to the culture medium were all found to have positive effects on green plant recovery (Zhou *et al.* 1991; Jacquard *et al.* 2009a; Sriskandarajah *et al.* 2015; Żur *et al.* 2015b). Even though no experimental proof has been published to date, the commonly accepted explanation of the beneficial effects on green plant recovery of such amendments, is that they simply increase the number of viable microspores containing non-gametophytic plastids.

Evidence of the effect of nuclear encoded genes on plastid development and albinism during in vitro androgenesis has been provided by means of genetic as well as gene expression studies. Albino plant recovery is a complex, recessive trait, for which both additive and non-additive effects as well as high and low heritabilities have been reported (Kumari *et al.* 2009). Molecular markers uniquely associated with albino production have been identified in rice, triticale and barley (He *et al.* 1998; Chen *et al.* 2007; Krzewska *et al.* 2015). Plastid development-related genes such a *DAG* homolog, essential for proplastid to chloroplast differentiation, were differentially expressed in genotypes producing many or few albino regenerants in barley (Muñoz-Amatriaín *et al.* 2009a). A study in wheat, where the albino and green regenerants themselves were compared, found that the 1892 identified genes were mainly related to components of the photosynthetic pathway, chlorophyll and porphyrin metabolism as well as the construction of thylakoids and the chloroplast envelope (Zhao *et al.* 2017).

From the above, it is clear that many factors can influence the frequency of albinism during in vitro androgenesis in the grass family. Exact mechanistic or genetic causes have not yet been conclusively identified. Nevertheless, targeted manipulations of ME induction methods may provide some measure of influence on this undesirable phenomenon.

1.4.5 Genetic control of androgenesis

Genotype-specific responses and genetic studies have indicated a role for nuclear encoded factors in the response to in vitro androgenesis (Dunwell 2010). It is generally assumed that a limited number of genetic loci with large effect separately control each of the distinct stages of successful androgenesis in the grass species: embryo production, plant regeneration and the ratio of green and albino plants. High heritability, often over 0.6, of most of these component traits of androgenic response has been reported (Lazar *et al.* 1984; Murigneux *et al.* 1994; Opsahl-Ferstad *et al.* 1994b; Moieni *et al.* 1997). Between one and four linkage mapping studies, using bi-parental populations of up to 100 individuals, have been performed in wheat, barley, rice, maize, oat and triticale (**Suppl.Table S1.1**). A large number of distinct genetic loci with an individual effect of 4-41% of the phenotypic variation of the component traits of androgenic capacity have been reported. The results of these studies are, however, not easily compared for the reasons already mentioned in section 1.4.3 ('Gene expression during microspore embryogenesis'). In addition, the diverse and sometimes imprecisely described methods used for the quantification of the androgenic response are an obstacle (Bolibok and Rakoczy-Trojanowska 2006; Seldimirova and Kruglova 2015). It is, for example, never reported whether multiple green shoots that often regenerate from a single embryo-like structure or callus in the less responsive species, are counted separately or together.

Even though several QTL of major as well as many QTL of minor effect have been reported, distinct QTL are detected by different groups and a general pattern is not obvious. Four QTL for green plant percentage were identified on wheat chromosomes 2AL, 2BL (2 QTL) and 5BL, for instance, which together explained 80% of variation in green plant percentage (Torp *et al.* 2001). In a different study on wheat, however, two QTL on chromosomes 1B and 7B explained 53% of the variation of that same trait (Nielsen *et al.* 2015). Also, when androgenic capacity of 90 triticale DHs was assessed in January, April and again in October, over half of the total number of detected QTL was identified at only one time (Krzewska *et al.* 2012). Pleiotropic effects of loci are often reported as well. Both green and albino plant production have been associated with single QTL in, for example, wheat, oat and barley (Kiviharju *et al.* 2004; Muñoz-Amatriaín *et al.* 2008; Nielsen *et al.* 2015). Conversely, the separate genetic control of green and albino plant production was proposed by those finding only QTL associated with one of those two traits in triticale, barley and rice (He *et al.* 1998; González *et al.* 2005; Krzewska *et al.* 2015). In addition, authors reporting QTL for green (or albino) plant percentage, arguably capture two traits

in one while losing any quantification of actual yield. One can therefore appreciate the ambiguity associated with the interpretation and comparison of these types of studies.

1.5 Thesis outline

Here, the production of DHs by means of in vitro AC as well as the genetic control of androgenic capacity have been investigated in an important forage crop. An efficient DH production system will not only accelerate perennial ryegrass research but also breeding, by enabling homozygous line production without being hindered by this species' effective self-incompatibility system (Cornish *et al.* 1979). Because of the high levels of recalcitrance to in vitro DH induction of the majority of the perennial ryegrass germplasm, significant methodological or genetic improvements are required before any widespread application of this technique is feasible. The current work aims to enable such improvements by providing better understanding of the genetic control of androgenic capacity as well as via the identification of genetic loci associated with high responsiveness.

Before any experimental results are presented, however, the potential future applications of DH induction in perennial ryegrass research and breeding are explored in **chapter 2**. The use of DHs to purge deleterious alleles, develop mapping populations for genetic and genomic studies, enable powerful haplotype mapping approaches, fix mutations and transgenes as well as allow for hybrid cultivar development are discussed.

Given that the most recent scientific journal paper on perennial ryegrass in vitro AC was published in the previous century, the first priority was the establishment of an efficient technical procedure. **Chapter 3** describes a pilot experiment in which an AC protocol is successfully tested on responsive germplasm and a high number of green, diploid and homozygous regenerants are produced. Both protocol and plant material originated from a breeding programme of the Danish breeding company DLF A/S, which was aimed at improving DH induction capacity and prematurely terminated approximately two decades ago. Seed from a number of paircrosses between DLF genotypes exhibiting varying degrees of androgenic capacity were used for the main experiment of this investigation.

Chapter 4 details the responses of nine of the bi-parental populations mentioned above to in vitro AC. Large, genotype-dependent variation, within as well as between the populations, was observed and was indicative of the polygenic control of androgenic capacity. Insights into the genetic control of perennial ryegrass embryo production and plant regeneration are discussed. In addition, the presence of different alleles within each of the populations was established, which further confirmed the suitability of these genotypes for a mapping approach.

Chapter 5 describes the results of the genotyping-by-sequencing (GBS) and genome-wide association (GWAS) approach that was used to identify genetic loci that significantly influenced the androgenic responses of the multi-parental mapping population presented in the previous chapter. A putative major QTL for green plant regeneration was detected on perennial ryegrass linkage group 5. In addition, a large number of QTL were detected for traits such as embryo and albino plant production. Several intriguing candidate genes were predicted in the genomic regions significantly associated with the studied trait.

Finally, the implications of the results presented in this thesis are explored in **chapter 6**.

2. Haploid and doubled haploid techniques in perennial ryegrass (*Lolium perenne* L.) to advance research and breeding

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Abstract

The importance of haploid and doubled haploid (DH) techniques for basic and applied research, as well as to improve the speed of genetic gain when applied in breeding programmes, cannot be overstated. They have become routine tools in several major crop species, such as maize (*Zea mays* L.), wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.). DH techniques in perennial ryegrass (*Lolium perenne* L.), an important forage species, have advanced to a sufficiently successful and promising stage to merit an exploration of what their further developments may bring. The exploitation of both in vitro and in vivo haploid and DH methods to 1) purge deleterious alleles from germplasm intended for breeding, 2) develop mapping populations for genetic and genomic studies, 3) simplify haplotype mapping, 4) fix transgenes and mutations for functional gene validation and molecular breeding, and 5) hybrid cultivar development are discussed. Even with the comparatively modest budgets of those active in forage crop improvement, haploid and DH techniques can be developed into powerful tools to achieve the acceleration of the speed of genetic gain needed to meet future agricultural demands.

Abbreviations

AC	anther culture
DH	doubled haploid
CENH3	centromere-specific histone 3 variant
CRISPR	clustered regularly interspaced short palindrome repeats
DMSO	dimethyl sulfoxide
ELS	embryo-like structures
IMC	isolated microspore culture
MAS	marker-assisted selection
QTL	quantitative trait locus/loci
SI	self-incompatible/self-incompatibility
TALEN	transcription activator-like effector nuclease
TILLING	targeting induced local lesions in genomes

2.1 Introduction

Biomass, produced by agriculture, is humanity's main source of food, feed and functional materials such as fibre for cloth, construction wood and industrial starches. In the latter half of the previous century, our agricultural systems generated higher yields than ever before during the Green Revolution. Presently, driven by predictions of global population growth, changing environmental conditions and the claim to arable land made by the biofuel sector, it is crucial that the yields increase sharply once again (Tilman *et al.* 2011). A doubling of the speed of yield growth is necessary in order to be able to feed the world's population in 2050 (Fischer *et al.* 2014). Since the Green Revolution however, the agricultural paradigm has changed. More output with less input, in terms of agrochemicals, fertilizers and water, on the existing cultivated land area is the current mandate (Foley *et al.* 2011). Sustainability issues such as biodiversity conservation, maintaining ecological services and safeguarding soil fertility should be addressed by modern agriculture in order to achieve both global food security and environmental sustainability (Brummer *et al.* 2011).

Aside from the undesirable option of expanding the agricultural area, production increases can be achieved in two ways. Firstly, by optimizing management practices, for example through precision agriculture or increasing water and nutrient supply to marginal lands, the gap between attainable and actual yields may be decreased (Fischer and Edmeades 2010; Foley *et al.* 2011). The second and generally considered most sustainable way to increase outputs is via genetic crop improvements by plant breeding, raising potential yields (Stamp and Visser 2012; Fischer 2015). Perhaps even more importantly, plant breeding enables the integration of novel traits, which is essential in achieving yield stability in the changing climatic conditions we are facing (Tester and Langridge 2010). There has, therefore, been a shift in emphasis towards breeding for crop characteristics such as nutrient and water use efficiency, tolerance to drought or salt stress and the ability to produce high and stable yields under sub-optimal conditions (Dawson *et al.* 2015). The challenge for contemporary plant breeding is to not only integrate new traits into our crops, but to accelerate the genetic gain of its breeding programmes at the same time, in order to achieve a doubling in speed of yield increase.

The potential impact of haploid and doubled haploid (DH) techniques on improving the speed of genetic gain when applied in breeding programmes, as well as their importance and diverse applicability in basic and applied research, cannot be overstated and has been the subject of numerous reviews (Forster *et al.* 2007; Dunwell 2010; Seguí-Simarro 2010; Germanà 2011; Dwivedi *et al.* 2015). DH techniques have been and are being used to accelerate the breeding programmes of a range of crops, most notably maize (*Zea mays* L.) and barley (*Hordeum vulgare* L.) (Geiger and Gordillo 2009; Seguí-Simarro 2015). A plethora of in vivo and in vitro protocols exist to accommodate the widely varying degrees of response between species to DH induction (Seguí-Simarro 2010). Even so, a number of scientifically (*Arabidopsis thaliana*) and economically important species such as tomato (*Solanum lycopersicum* L.), cotton (*Gossypium* spp.), grape (*Vitis* spp.), trees and medicinals are still considered recalcitrant (Dwivedi *et al.* 2015).

This review aims to illustrate the importance of allocating time and resources towards further developing the efficiency and efficacy of haploid and DH techniques for forage crop breeding. In forage-based agriculture, to which close to 70% of the world's agricultural land is devoted (FAOstat, 2013), the challenges described above are no less pressing (Smith and Spangenberg 2014). This extensive area is not only at the basis of global meat and milk production, but also plays a major role in ecosystem processes such as nutrient cycling and carbon sequestration as well as being a reservoir for the preservation of biodiversity (Reheul *et al.* 2010). Technologies such as DH induction can play a key role in accelerating breeding of forages, which we will illustrate using the economically important crop perennial ryegrass (*Lolium perenne* L.) as an example.

2.2 Haploids and doubled haploids: their production and use in breeding and research

Haploids are defined as plants with a single chromosome set (*n*) and DHs as 100% homozygous individuals stemming from chromosome doubled haploids (2*n*). The preferred method for the production of haploid or DH plants differs per species and depends on protocol availability as well as efficiency in terms of investments and yields. Immature microspores, which are abundant in most flowering plants, can be induced to develop into embryos and subsequently into plants in vitro (androgenesis) (Seguí-Simarro and Nuez 2008; Ferrie and Caswell 2011). Isolated microspore culture (IMC), although technically more challenging, is preferred over anther culture (AC) because of its higher efficiency (Ferrie and Caswell 2011) and has been routine in barley, tobacco (*Nicotiana tabacum* L.) and rapeseed (*Brassica napus* L.) breeding for some time (Dwivedi *et al.* 2015). Response to in vitro DH induction is highly genotype dependent and colchicine can be needed for chromosome doubling (Seguí-Simarro 2010). Additionally, factors such as donor plant growing conditions, stress pre-treatment, medium composition and culture conditions all influence the embryo induction rates, number of regenerated plants and, especially in Poaceae species, the ratio between green and albino regenerants (Shariatpanahi *et al.* 2006; Kumari *et al.* 2009; Seguí-Simarro 2010). After roughly 50 years of research into DH induction methods it must be concluded that there probably is no single 'master switch' to stimulate the formation of embryos from any species of microspore (Seguí-Simarro and Nuez 2008). Successful protocols therefore differ significantly between, and even within, species or are not yet available at all (Maluszynski *et al.* 2003; Touraev *et al.* 2009; Germanà and Lambardi 2016).

Haploid seed production can be induced in vivo by using irradiated or heat treated non-functional pollen, pollen of distantly related species followed by uniparental genome elimination (wide hybridization) or pollen from haploid inducer genotypes (Dunwell 2010). DH wheat (*Triticum aestivum* L.) plants, for example, can be efficiently produced via wide crosses with maize, embryo rescue and chemical chromosome duplication (Niu *et al.* 2014). In hybrid maize breeding, haploid inducer lines are routinely used to obtain an average of 10% haploid kernels on the seed parent. The resulting haploid seedlings are treated with colchicine to obtain DHs (Geiger and Gordillo 2009). Ovule culture (gynogenesis) is mainly used in species recalcitrant to androgenesis, since its efficiency is much lower due to the smaller number of ovules available per flower. The value of the DH can make gynogenesis an economical option however, for example in sugar beet (Beta vulgaris L.), onion (*Allium cepa* L.) and some tree species (Chen *et al.* 2010).

Haploid and DH technologies have found wide application, especially in the field of plant breeding, for those crops where protocols are sufficiently effective (Pink *et al.* 2008). Major reductions in the time needed for cultivar development have been realized, since the availability of DHs eliminates the need for the 5-7 generations of selfing traditionally required to produce inbred lines (Dwivedi *et al.* 2015). In combination with marker-assisted selection (MAS), DH induction has significantly increased the efficiency of backcross breeding (Tuvesson *et al.* 2006). By applying DH induction to one of the early backcross generations, genotypes carrying the trait to be introgressed as well as having the highest possible proportion of the elite genome can be selected quickly. DHs have been released directly as cultivars in barley (Devaux and K.J. Kasha 2009), rice (*Oryza sativa* L.) (Mishra and Rao 2016), rapeseed (Ferrie and Möllers 2010), wheat (Niu *et al.* 2014) and other crops, or used as parents of F1 hybrids of vegetables and maize (Geiger and Gordillo 2009), in order to benefit from hybrid vigour (heterosis) (Lippman and Zamir 2006; Birchler *et al.* 2010). In ornamental breeding, haploid plants have commercial value of their own because of their smaller size compared to diploids (Ferrie 2012).

Furthermore, DH populations have been invaluable for QTL discovery, especially in cereals, since their immortality enables robust phenotyping data to be gathered in different locations and over several years (Obsa *et al.* 2016). In outcrossers which suffer from inbreeding depression, using at least

one DH parent to create mapping populations has been effective (Germanà 2011). Genome sequencing studies have used haploids or DHs to reduce the complexity of assembly, for example in peach (*Prunus persica*), citrus (*Citrus* spp), coffee (*Coffea* spp), apple (*Malus pumila*) and pear (*Pyrus* spp) (Dunwell 2010). Microspores of tobacco, rapeseed, wheat and barley are exploited in transformation and mutagenesis programmes, in order to fix mutations and transgenes in a single step through subsequent DH induction (Kumlehn *et al.* 2006; Brew-Appiah *et al.* 2013; Kapusi *et al.* 2013; Shen *et al.* 2015; Huang and Liu 2016). For example in *Brassicas*, microspore mutation studies have enabled modifications of disease resistance, cold tolerance and fatty acid composition (Ferrie and Caswell 2016). Also, in vitro microspore culture systems have allowed for detailed study of embryogenesis, early cell fate decisions, embryogenesis and totipotency (Soriano *et al.* 2013; Daghma *et al.* 2014; Seifert *et al.* 2016). All of these applications of haploid and DH techniques, as well as many others not mentioned here, could confer the same benefits to perennial ryegrass breeding and research as they have done and currently do in species for which effective and efficient DH induction protocols are available (Dwivedi *et al.* 2015).

2.3 Perennial ryegrass

2.3.1 Perennial ryegrass breeding

Perennial ryegrass, the economically most significant forage grass worldwide, is popular for its good yields, high digestibility for animals and excellent grazing tolerance. Natural populations are diploid (2*n* = 2x =14) but both diploid and artificially created tetraploid cultivars are available. Compared to diploids, tetraploid plants are larger, have a higher nutritive value for animals and better abiotic and biotic stress tolerances but reduced sward density and lower persistence (Smith *et al.* 2001, 2003; Nair 2004). In contrast to the thousands of years of breeding effort in annual grasses such as wheat, barley and rice, perennial grass breeding is not even a century old (Wilkins 1991). Nevertheless, important improvements in yield potential, persistency and disease resistance, as well as feeding value – through increased water-soluble carbohydrate content and reduced aftermath heading for example – have been achieved (Humphreys 2005; Sampoux *et al.* 2011). The allogamous nature of this species, due to its highly effective gametophytic self-incompatibility system (Cornish *et al.* 1979), has until now restricted breeding to the population level, resulting in marked genetic diversity and heterozygosity within cultivars (Blackmore *et al.* 2016). Modern perennial ryegrass varieties are synthetic populations, selected from the progeny of a polycross between elite genotypes, obtained by recurrent selection, with a good agronomical performance (Wilkins 1991). As a consequence, genes governing key agricultural traits are rarely completely fixed and cultivar characterization for variety registration purposes is complicated (Wang *et al.* 2014).

Compared to cereal grain yield increases, perennial ryegrass yield gains have been described as low to non-existent (Humphreys 2005). Commonly cited causes for this relatively slow progress, calculated as an increase in dry matter yield of just 3.2% per decade (Sampoux *et al.* 2011), are 1) the longer breeding cycle of perennial forage crops, 2) the absence of a harvest index trait to facilitate partitioning of dry matter into the marketed product, 3) a lack of commercial exploitation of heterosis, 4) a focus on breeding for other traits than yield, such as resistance to crown rust (*Puccinia coronata*), reduced aftermath heading and early spring growth (Casler and Brummer 2008). In effect, the modest yield increases in perennial ryegrass are most likely due to constraints stemming from life history traits and the techniques available to forage breeders rather than physiological limitations or lack of genetic variation (Sampoux *et al.* 2011). If genetic gains are to be improved, it is therefore imperative to expand the arsenal of breeding tools as well as to find ways of working with or around the characteristic life cycle of perennial ryegrass. As has been shown in other crops, DH techniques have excellent credentials to help address both of these challenges (Dwivedi *et al.* 2015).

2.3.2 Doubled haploids in perennial ryegrass

Perennial ryegrass DH production through AC (**Figure 2.1**) was first attempted in the late 1970s. Initially, only embryos and albino plants were obtained, until the first green regenerants were reported in 1984 (Stanis and Butenko 1984). During the following decades, contemporary protocols for barley (Boppenmeier *et al.* 1989) and wheat (Olesen *et al.* 1988) AC were adapted for use in perennial ryegrass by optimizing the pre-culture temperature stress, in vitro carbohydrate source, growth regulator additions and culture conditions such as light and temperature (Boppenmeier *et al.* 1989; Bante *et al.* 1990; Creemers-Molenaar and Beerepoot 1992; Opsahl-Ferstad *et al.* 1994a). The total number of regenerated plants was thus increased, although the percentage of albinos remained high and genotypes capable of producing green plants by androgenesis were described as being rare exceptions (Olesen *et al.* 1988; Madsen *et al.* 1995). For example, only 71 out of 229 genotypes, derived from 15 cultivars, produced regenerants after DH induction and only one genotype produced green plants (Hussain *et al.* 2006). Only one study recounts green plant regeneration via IMC in perennial ryegrass, while in vitro gynogenesis or in vivo haploids have never been reported (Andersen *et al.* 1997).

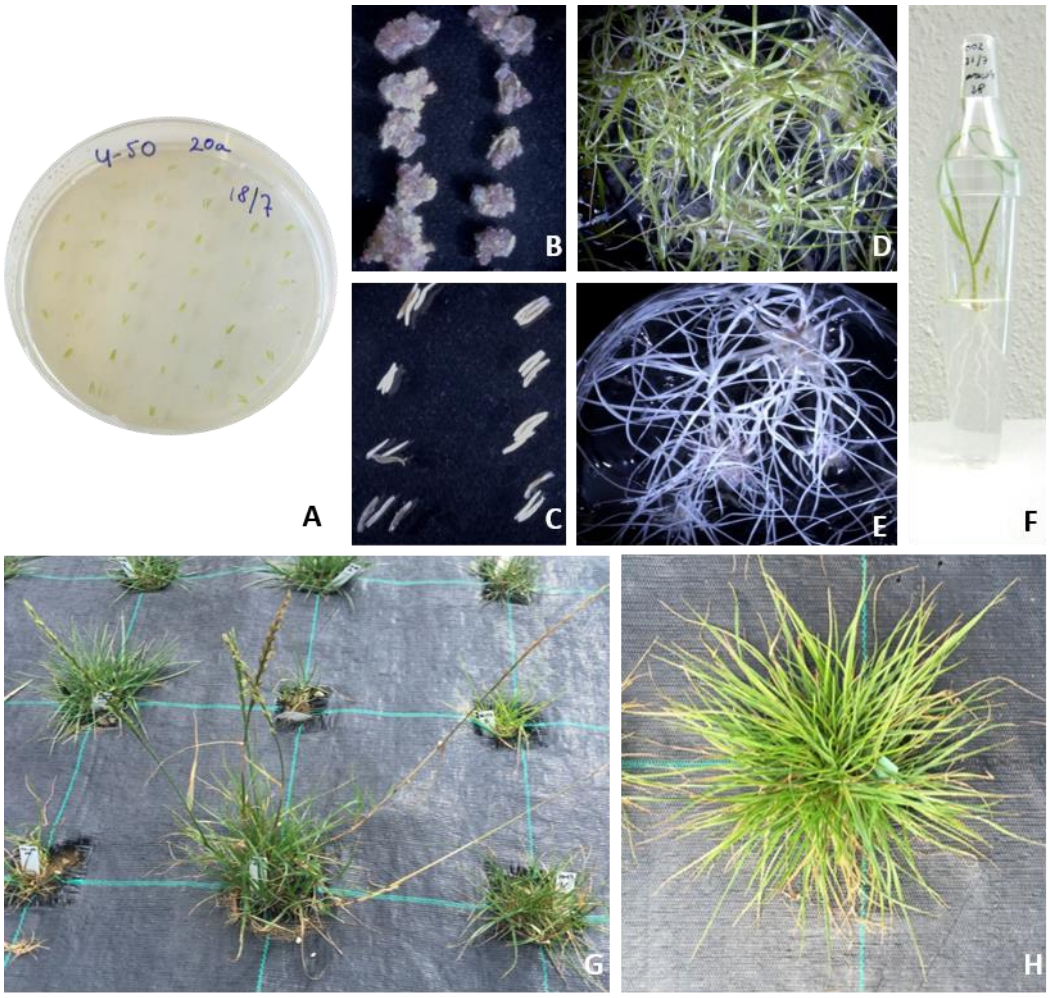

Figure 2.1 Different stages of perennial ryegrass (*Lolium perenne* L.) anther culture (AC) and doubled haploid (DH) production. **A** – Fresh anthers on DH induction medium; **B** – Anthers of a responsive donor genotype producing many embryo-like structures (ELS) on DH induction medium, six weeks post AC; **C** – Anthers of an unresponsive donor genotype, six weeks post AC; **D** – Green and albino putative DH plantlets growing from ELS cultured on DH regeneration medium, four weeks post subculture; **E** – Albino plantlets growing from ELS, four weeks post subculture; **F** – Putative DH plantlet on

regeneration medium; **G** – Flowering DH plant in the field; **H** – Vigorous vegetative DH plant in the field (photographs by Begheyn, R. F.)

As in other crops, the ability to 1) form embryos or embryo-like structures (ELS), 2) regenerate plants, and 3) the ratio of green and albino regenerants, is under genetic control in perennial ryegrass (Olesen *et al.* 1988; Bante *et al.* 1990; Halberg *et al.* 1990; Madsen *et al.* 1995). Anther donor genotype was found to determine 73% of the variation in embryo yield (Olesen *et al.* 1988) and 80% of the variation in green plant production (Madsen *et al.* 1995). Evidence of transgressive segregation was reported when a 60% higher green plant yield, up to 59 green plants per 100 anthers, was observed in the progeny of crosses between three genotypes responsive to AC (Halberg *et al.* 1990; Opsahl-Ferstad *et al.* 1994b). Based on these results, Halberg *et al.* proposed the so-called 'inducer approach': introducing the ability to produce high numbers of green plants into recalcitrant germplasm by crosses with genotypes exhibiting that ability (the inducers; not to be confused with in vivo haploid inducers) (Halberg *et al.* 1990). Evidence for the efficacy of the inducer approach was reported by Madsen *et al.*, who achieved a 7.3 fold increase in green plant yield in crosses between inducers and regular breeding germplasm (Madsen *et al.* 1995). The inducer approach was also effective in darnel ryegrass (*L. temulentum* L.) where, using an adapted perennial ryegrass AC protocol, a higher responsiveness was achieved through intercrossing good responders (Wang *et al.* 2005).

Androgenic capacity is recessively inherited in perennial ryegrass and probably controlled by a relatively small number of genes with a large effect (Madsen *et al.* 1995), similar to what is supposed to be the case in barley (Chen *et al.* 2007). The ability to develop ELS seems to be controlled by additive gene effects, while total plant regeneration capacity and green plant percentage seem to be controlled by dominance (Opsahl-Ferstad *et al.* 1994b). There may be different genes with epistatic interactions affecting green and albino plant production (as was found in wheat; Agache *et al.* (1989)). However, since it has been hypothesised that in vitro conditions cause the mutations that lead to plastid development defects, albinism rates are likely to be influenced more by environment than by genetics (Opsahl-Ferstad *et al.* 1994b; Torp and Andersen 2009). To date, no QTL studies on androgenetic capacity in perennial ryegrass have been published. In the cereals, a limited number of reports exist, typically finding between 1 and 8 QTL for each component trait, explaining 3-65% of the variation (Bolibok and Rakoczy-Trojanowska 2006). For example, two QTL, explaining 53% of the variation in green plant regeneration, were recently found in wheat (Nielsen *et al.* 2015). In a triticale (x *Triticosecale*) QTL study on androgenic responsiveness, 28 QTL on 5 chromosomes were found, explaining 5-22% of the phenotypic variation (Krzewska *et al.* 2012).

Relatively high spontaneous chromosome doubling rates, between 50 and 80%, are common in perennial ryegrass AC (Olesen *et al.* 1988; Bante *et al.* 1990; Halberg *et al.* 1990; Begheyn *et al.* 2016b). Colchicine, a toxic chromosome doubling agent, is therefore usually not applied, making the whole procedure safe for human health (Melchinger *et al.* 2016). Isozyme pattern characterization studies have confirmed that nearly all diploid regenerants are in fact homozygous. Andersen *et al.*, for example, reported that only 3 out of 913 anther derived plants were heterozygous (Andersen *et al.* 1997). Non-diploid regenerants are mostly haploids but also include a small percentage of homozygous polyploids. Apart from ploidy level, confirmation of homozygosity and reports of obvious signs of inbreeding depression, there is a marked lack of information on the performance of the DH plants. One field study compared the biomass and seed yields of DH lines and their parents, finding reductions of 80 and over 90%, respectively (Opsahl-Ferstad 1993). A different field trial evaluated seed set in 75 DH lines selected for their vigorous growth and found a 70% lower performance compared to their parents (Andersen *et al.* 1997). Nevertheless, one DH clone produced 5.08 g seeds/plant compared to the 1.44 g of its parent, suggesting that it is possible to identify DH plants with both vigorous growth and

excellent fertility. Self-fertility of DH plants was investigated in a study by Madsen *et al.* and seed set was found to be very low at 0-0.4 seeds per spike (Madsen *et al.* 1993).

2.4 Future applications of haploid and doubled haploid techniques in perennial ryegrass

Strategies to accelerate perennial ryegrass breeding in order to increase yield gains have been and are being discussed (Casler and Brummer 2008; Conaghan and Casler 2011; Arias Aguirre *et al.* 2011; Wang and Brummer 2012; Manzanares *et al.* 2016b), but the role that DH techniques could play in this context has hardly ever been considered. DH techniques have the potential to help unlock and increase the genetic variation available for selection as well as facilitate the development of more rapid and efficient selection and breeding procedures (Langridge and Fleury 2011). An effective perennial ryegrass AC protocol is available, however, transitioning to IMC would certainly increase efficiency and yield even further. Major leaps in efficiency are likely to be achievable for both methods, since adaptations which increase the yields of already highly successful protocols continue to be published regularly (Seguí-Simarro 2010; Sinha and Eudes 2015; Sriskandarajah *et al.* 2015). Only this year for example, a two to four fold increase in the number of embryos and regenerated plants in both barley and wheat was reported, achieved through the addition of dimethyl sulfoxide (DMSO) to the pre-treatment medium (Echávarri and Cistué 2016).

In vivo haploid induction has never been reported in perennial ryegrass even though, due to low genotypic specificity and relatively simple technical demands, it could prove highly practical. Additionally, segregation distortion due to in vitro androgenesis, resulting in higher allele frequencies of the donor most amenable to tissue culture, can thus be avoided (Hayward *et al.* 1990; Devaux and Zivy 1994; Pink *et al.* 2008; Muñoz-Amatriaín *et al.* 2008; Zhang *et al.* 2011; Bélanger *et al.* 2016). Recently, two DH inducer lines of annual ryegrass (*L. perenne* L. subsp. *multiflorum* (Lam.) Husnot [syn. *L. multiflorum* Lam.]) have been registered which, when crossed with tall fescue (*L. arundinaceum* (Schreb.) Darbysh. [syn. *Festuca arundinacea* Schreb.]), produce F1 hybrids that can yield low percentages of both tall fescue and annual ryegrass DHs (Kindiger 2016). Selection could be performed on the vigorous F1 hybrids prior to inflorescence harvest and the subsequent recovery of 1-5 DHs per plant (Kindiger 2012). Perhaps this technique could be applied to perennial ryegrass as well. Alternatively, it might be worthwhile to investigate the existence of perennial ryegrass haploid inducing genes, such as CENH3 discovered in Arabidopsis (Ravi and Chan 2013), which seems to also affect centromere disruption and genome elimination in maize (Kelliher *et al.* 2016), barley and sugar beet (Karimi-Ashtiyani *et al.* 2015).

Dwivedi *et al.* have recently published a comprehensive review of the myriad applications of DHs in plant breeding and research (Dwivedi *et al.* 2015), many of which may be applicable in perennial ryegrass as well. However, it is important to realize that the costs associated with the implementation of the DH applications which have been proposed, or indeed are already applied elsewhere, may be prohibitive in a forage crop. The significantly lower economic value of perennial ryegrass compared to crops such as maize, barley and rice, limits the available resources for using and further developing DH techniques. Nevertheless, there definitely are exciting possibilities for exploiting DHs within the budgetary reach of perennial ryegrass researchers and breeders, some of which we will highlight in this section.

2.4.1 Purging deleterious alleles

Recessive deleterious mutations are thought to play a major role in inbreeding depression, which is observed when allogamous species, such as perennial ryegrass, are selfed (Charlesworth and Willis 2009). Such recessive alleles are masked in heterozygous genotypes, but are carried at one or more loci in a majority of gametes. To purge deleterious alleles from germplasm intended for breeding, DH induction has been proposed as the most effective method. By fixing maize landraces as DH lines, the

genetic basis of elite maize germplasm could be broadened (Wilde *et al.* 2010). In one field study for example, genetically highly diverse and distant lines with a grain yield similar to elite lines could be selected (Strigens *et al.* 2013). The introgression of interesting traits from landraces without incurring yield impairments is thus enabled by using a DH selection phase. Introgression breeding is commonly practiced in the *Lolium-Festuca* complex as well, for example to improve winter hardiness, crown rust resistance and drought tolerance (Humphreys 2005). A recent study revealed extensive genetic variation in European ecotypes of perennial ryegrass, much of which has not yet been exploited in modern cultivars (Blackmore *et al.* 2016). Germplasm intended for population and synthetics breeding can benefit from the inclusion of a DH step to reduce deleterious genetic load. Given the status of DH techniques in perennial ryegrass, purging deleterious alleles from natural populations for introgression breeding is not feasible using current in vitro methods. The development of an in vivo haploid induction system would be exceedingly useful for work with natural populations of the *Lolium-Festuca* complex.

2.4.2 Doubled haploids for genetics and genomics

To accelerate perennial ryegrass breeding, increased knowledge on the genetics underlying traits of interest allows for a more informed and thus effective selection process to exploit available genetic variation. DH technologies can significantly reduce the time and costs required for genomics and genetics studies of perennial ryegrass, as it has done in other crops (Dunwell 2010; Dwivedi *et al.* 2015). Since the existing DH induction procedure is not yet successful in all genotypes, it may be sensible to restrict some of the methods proposed in the following section to a few 'model genotypes' at this time. These genotypes should be selected for their ability to produce large numbers of green, chromosome doubled, vigorous and fertile DHs. Similar to the highly androgenetic barley cultivar 'Igri' (Larsen *et al.* 1991; Kumlehn *et al.* 2006; Muñoz-Amatriaín *et al.* 2008; Jacquard *et al.* 2009b; Gurushidze *et al.* 2014), such model genotypes may yield a wealth of information which can then be used in perennial ryegrass breeding.

2.4.2.1 Transformation and mutation

An efficient transformation system is an important tool for functional gene validation and molecular breeding. Protocols to transform (embryogenic) calli using *Agrobacterium*, particle bombardment and electroporation have been published and successfully used in perennial ryegrass (reviewed in Giri and Praveena (2014)), although problematically low regeneration efficiencies are often reported. Additionally, it is challenging to perform the selfings needed in order to fix a transgene for further evaluation in this SI species. In vitro or in vivo microspore transformation, either by *Agrobacterium*, particle bombardment or cell-penetrating peptides (CPPs), and subsequent DH induction and regeneration can overcome both these issues (Eudes *et al.* 2014). *Agrobacterium*-mediated transformation was successfully used in concert with IMC in barley and resulted in single copy (Kumlehn *et al.* 2006) and even transcription activator-like effector nuclease (TALEN) mediated gene knock-out DH transformants (Gurushidze *et al.* 2014). Similar successes were reported in other crops, such as wheat (Brew-Appiah *et al.* 2013) and rapeseed (Ferrie and Möllers 2010). Linear DNA, enzymes and proteins could be delivered into triticale (Chugh *et al.* 2009) and wheat (Bilichak *et al.* 2015) microspores by CPPs. These types of peptides can thus be used for both transformation and transgene-free genome editing, if for example proteins and guide RNAs of the CRISPR/Cas system are introduced (Kumar and Jain 2015).

Mutation techniques combine well with DH technology, because, as with transgene approaches, homozygous and stable integration of (recessive) mutations does not require additional generations of selfing (Maluszynski *et al.* 1995). Additionally, DHs or their microspores are excellent targets for mutagenesis since the absence of background variation in their genomes allows for easy identification of mutants. Both seeds derived from DHs as well as haploid microspores have been mutagenized in a

range of crop species, such as wheat, barley and rice (Szarejko and Forster 2007). A number of microspore mutagenesis studies have been performed to target oil quality characteristics in several *Brassica* species and significant diversity could be induced for these traits (Ferrie and Caswell 2016). The detection of false-positives can be prevented by using DH starting material for TILLING experiments, a technique that has recently received attention to advance forage grass breeding (Manzanares *et al.* 2016b). In barley, seeds of a DH line were used for a TILLING experiment (Kurowska *et al.* 2012), however, DH derived microspores would be a better target in perennial ryegrass in order to circumvent the need for selfing.

Combining mutagenesis and transformation studies with DH techniques significantly reduces the time and costs required to obtain modified homozygotes for genotype-phenotype validation as well as generate and fixate genetic variation (Shen *et al.* 2015). Markedly smaller populations are required to obtain genotypes with multiple homozygous transgene inserts or mutations when DH induction is used instead of self-fertilization (Lübberstedt and Frei 2012). Considerable reductions in space can be gained if the selection of regenerants can be done during the in vitro stage. Small tissue samples for DNA extraction and further analysis may be taken from in vitro cultures, for example to detect genotypes with modifications to the gene of interest in reverse genetics screens. Alternatively, a selection agent, such as NaCl or a pathogen-derived substance, can be added to the culture medium after mutagenesis, in order to obtain DH mutants with tolerances to certain stresses (Germanà 2011).

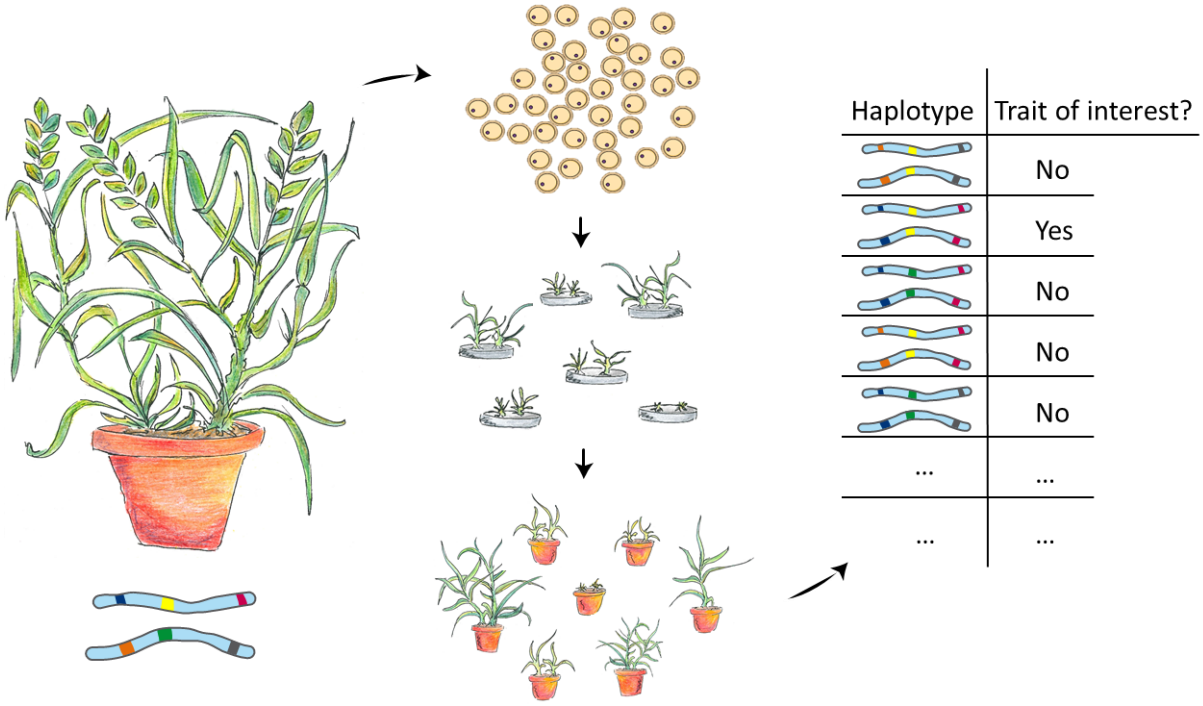

Figure 2.2 Schematic representation of a direct haplotype mapping strategy by DH induction of microspores derived from a single, heterozygous genotype exhibiting a trait of interest. Through recombination during meiosis in the donor plant, the microspore population represents a large diversity of possible haplotypes. Both donor and microspore-derived DH plants are genotyped and phenotyped so that statistical methods can be used to infer the haplotype associated with the trait of interest (in this case the dark blue, yellow and red alleles).

2.4.2.2 Population development for mapping

Chromosome maps as well as a vast number of mapped genetic markers have been established through DH techniques in a range of species, such as rapeseed (Delourme *et al.* 2013), wheat (Cabral *et al.* 2014) and barley (Sannemann *et al.* 2015). Indeed, the genetic map of the International *Lolium*

Genome Initiative (ILGI) was constructed from a cross between a DH and a heterozygous perennial ryegrass genotype (Bert *et al.* 1999; Jones *et al.* 2002). Segregating DH populations provide excellent opportunities to find marker-trait associations through linkage mapping and have been extensively used in many crops (Guo *et al.* 2013). Tuvesson *et al.* describe how a DH mapping population for marker-trait associations can be created and maintained in rye, a crop which, like perennial ryegrass, suffers from inbreeding depression (Tuvesson *et al.* 2006). Two distinct DHs are used to produce an F1 population, individuals of which are then subjected to DH induction. Both the parental DHs and the F1-derived DHs are crossed to a tester in order to keep them alive. *Lolium-Festuca* hybrids exhibit high levels of recombination within their gametes and thus offer unique opportunities to determine genome organization, elucidate genetic control of key agricultural traits and map markers (King *et al.* 2007). Such introgression mapping combines well with DH induction and this approach has already been successfully used to obtain and select useful gene combinations for freezing-tolerance (Humphreys *et al.* 2007).

Inducing a large number of DHs from a single, highly heterogeneous genotype could circumvent the construction of a designed population altogether and allow for direct haplotype mapping (**Figure 2.2**). Every single microspore-derived plant is a unique product of recombination between the chromosomal pairs of the donor and an analysis of the inheritance of markers and genes is therefore possible in such a population. Single pollen grain PCR-based sequencing methods for recombination studies have been described in barley, maize, sorghum and other crops (Petersen *et al.* 1996; Chen *et al.* 2008; Dreissig *et al.* 2015). However, a major advantage of DH induction over these approaches is that it allows for phenotyping in addition to genotyping. Additionally, a sufficiently high number of DHs can be regenerated from the microspores of a single plant to allow for fine-mapping or even map-based cloning approaches, without being dependent on seed set of specific crosses.

Table 2.1 Comparison of three methods to obtain inbred or 100% homozygous lines in perennial ryegrass.

	Repeated self-fertilization	In vitro doubled haploid induction	In vivo doubled haploid induction
Method available	yes	yes[1]	no
Genotype specificity	low	high	unknown
Efficiency	low	high[2]	unknown
Required skill	low	moderate	low[3]
Space required	high	low	high
Lab requirements	none	high	low
Generations required	5-6[4]	1	1
Diploid regenerants	100%	50-80%	unknown[5]
Obstacles	self-incompatibility, inbreeding depression	albinism, inbreeding depression	inbreeding depression
Side effects of procedure[6]	allows selection every generation	gametoclonal variation, somatoclonal variation, ploidy level variation, segregation distortion	part of inducer genome could integrate

[1] Anther culture, possibly isolated microspore culture.
[2] Up to several hundreds of plants per 100 cultured anthers using current anther culture techniques.
[3] High if embryo rescue is required.
[4] Some residual heterozygosity (theoretically 1.6% after 6 generations).
[5] If the chosen method generates haploids, colchicine will be needed to double the chromosomes.
[6] Can be positive or negative depending on end-use.

2.5 Doubled haploids for hybrid breeding

Hybrid breeding has made a significant contribution to the acceleration of yield gains of many important crops through the exploitation of the phenomenon of heterosis (Goff 2011). In perennial ryegrass, DH induction may well be the most practical method for the development of homozygous lines for hybrid breeding (**Table 2.1**). Currently, most breeding germplasm is recalcitrant to DH induction, necessitating the introgression of androgenic capacity (Halberg *et al.* 1990; Madsen *et al.* 1995). Using modern mapping approaches, it should be feasible to obtain markers associated with the few major genes expected to control ELS formation and green plant regeneration (Opsahl-Ferstad *et al.* 1994b; Madsen *et al.* 1995), in order to accelerate their introgression into elite material (Bolibok and Rakoczy-Trojanowska 2006).

Even though inbreeding depression is severe in perennial ryegrass DHs, reports of vigorous and fertile plants do exist (Andersen *et al.* 1997). In the early days of maize (Hallauer *et al.* 2010) and rye hybrid breeding (Geiger and Miedaner 2009), both allogamous cereals suffering from inbreeding depression as well, selection among inbred lines was successfully used to improve their vigour and fertility to economically practical levels. Similar to the purging of deleterious alleles described above, DH induction may thus be used as a selection tool against inbreeding depression (Niels Roulund, personal communication; Charlesworth and Willis (2009)). Also, negative effects from inbreeding depression may be averted if diploid or tetraploid single cross hybrids are used as parents to produce double cross hybrids. Indeed, tetraploid cultivars are of special interest in the context of hybrid breeding, because polyploids often exhibit progressive heterosis the larger the genetic diversity between their component genomes is (Brummer 1999; Birchler *et al.* 2010). A single cross hybrid between two homozygous autotetraploids (AABB) will display heterosis, but a double cross hybrid from two distinct single cross hybrids (ABCD) is nearly always more heterotic (Riddle and Birchler 2008). Conversely, tetraploid *Lolium-Festuca* hybrids have been converted into diploids by AC to reduce vigour and plant size for turf applications (Kopecky *et al.* 2005). DH techniques can thus allow breeders to manipulate ploidy level and homozygosity in order to maximize the exploitation of heterosis in future perennial ryegrass cultivars.

Hybrid seed production requires efficient multiplication of inbred lines as well as an effective method to control pollination. Elucidation of the SI system and the development of markers for its components are now within reach (Yang *et al.* 2009; Klaas *et al.* 2011; Manzanares *et al.* 2016a) and should enable maintenance and multiplication of DH lines through seed. Interestingly, repeated selfing of DHs has been proposed as a method to cause the breakdown of SI, since rare mutations in SI genes in pollen grains can thus be selected (Madsen *et al.* 1993). Schemes to produce F1 seed of perennial ryegrass based on population hybridization (Wilkins 1991; Brummer 1999), cytoplasmic male sterility (CMS) (Arias Aguirre *et al.* 2011; Islam *et al.* 2014) and SI (Arias Aguirre *et al.* 2011; Pembleton *et al.* 2015; Do Canto *et al.* 2016) have been proposed (see also Posselt (2003)), although opinions differ on which method is the most practically and economically feasible.

2.6 Concluding remarks

DH techniques in perennial ryegrass have advanced to a sufficiently successful and promising stage to warrant a tentative glance at what future developments in this field may bring to both breeding and research (Forster *et al.* 2007). Some exceedingly useful applications require the realization of relatively small improvements to existing in vitro protocols, which could be derived from successful work in barley, rye and wheat (Echávarri and Cistué 2016). For example, highly androgenic genotypes may be used as models in DH or microspore transformation and mutagenization studies or for direct haplotype mapping (Kumlehn *et al.* 2006). Homozygous line production for hybrid breeding, however, will require either improved in vitro protocols that are effective in recalcitrant genotypes or the introgression of

androgenic capacity into breeding germplasm (Halberg *et al.* 1990; Madsen *et al.* 1995). Additionally, investigations into and improvement of the agronomic performance of perennial ryegrass DHs, as well as an efficient seed production system, are required for an economically feasible production of hybrid cultivars. An alternative strategy to significantly reduce genotype-specific responses to in vitro DH production, would be the development of in vivo haploid or DH inducers (Kindiger 2016). These would also allow the purging of deleterious alleles from natural populations, thus enable a broadening of the genetic variation available for breeding without incurring high levels of performance impairment (Strigens *et al.* 2013).

All of the haploid and DH techniques discussed here should reduce the time, space and investment required to perform effective perennial ryegrass research and breeding. Since this species can be regarded as a model for other grasses, any progress made should be beneficial to them as well. Even with the comparatively modest budgets of those active in forage crop improvement, haploid and DH techniques can be developed into powerful tools to achieve the acceleration of the speed of genetic gain needed to meet future agricultural demands.

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3. Efficient doubled haploid production in perennial ryegrass (*Lolium perenne* L.)

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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, which is 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. It is therefore difficult in practical terms to develop homozygous inbred lines through 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. We aim 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.

3.1 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 its self-incompatibility (SI) system 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. The 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 by means of 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 that is 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 during 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 in addition be genotyped via 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 androgenesis (Guo *et al.* 2013).

We aim 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 made significant progress recently (Studer *et al.* 2012; Islam *et al.* 2014). Practical and economical systems for both 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.

3.2 Material and Methods

3.2.1 Plant material

DLF Trifolium A/G, Denmark, kindly provided the 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, determined by microscopy, were harvested in May-June 2014 for use in the doubled haploid induction experiment.

3.2.2 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) where included in the induction medium, kinetin and 1-naphthaleneacetic acid (NAA) 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 minute 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.

3.2.3 Flow Cytometry

Leaf material of a subset of the green putative DH plants was harvested so that their DNA content could be estimated with flow cytometry. A genotype of the cultivar 'Arara' was used as a diploid control so that the ploidy level of the putative DH plants could be determined.

3.3 Results and Discussion

3.3.1 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 3.1**). 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).

Table 3.1 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. PP – polycross progeny.

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

Genotype	Number of ELS per 100 anthers	Number of plants per 100 anthers	Percentage albino plants	Percentage green plants
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			

3.3.2 Flow Cytometry

In perennial ryegrass anther culture, the genome 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 3.2**). 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 3.1**). However, PP 007 had a chromosome doubling efficiency of 33% compared to 50% for PP 012.

Table 3.2 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. PP – polycross progeny.

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	

3.4 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. Intriguing variation for component traits of androgenetic capacity was observed between the genotypes. The ploidy level analysis still needs to be complemented with a marker assay to determine whether the diploid genotypes are actual DH plants in order to draw clear conclusions on the outcome of our experiment. These 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, so that the DHs 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 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, as well as the foreseen developments in grass CMS research, will bring us closer to the reality of hybrid grass cultivars than ever before.

4. 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.

The responses of nine bi-parental populations, segregating for microspore embryogenesis and plant regeneration capacity, to an effective AC protocol are reported. 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.

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

4.1 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 programmes. 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 programmes, 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 K.J. Kasha 2009; Niu *et al.* 2014; Mishra and Rao 2016; Naik *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 (Begheyn *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 pre-treatment 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 programmess (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 programmess 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.* 2009a; Schedel *et al.* 2017), 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 (Begheyn *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.

4.2 Materials and Methods

4.2.1 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 programme. 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 **Figure 4.1**. 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 (**Suppl. Table S4.1**).

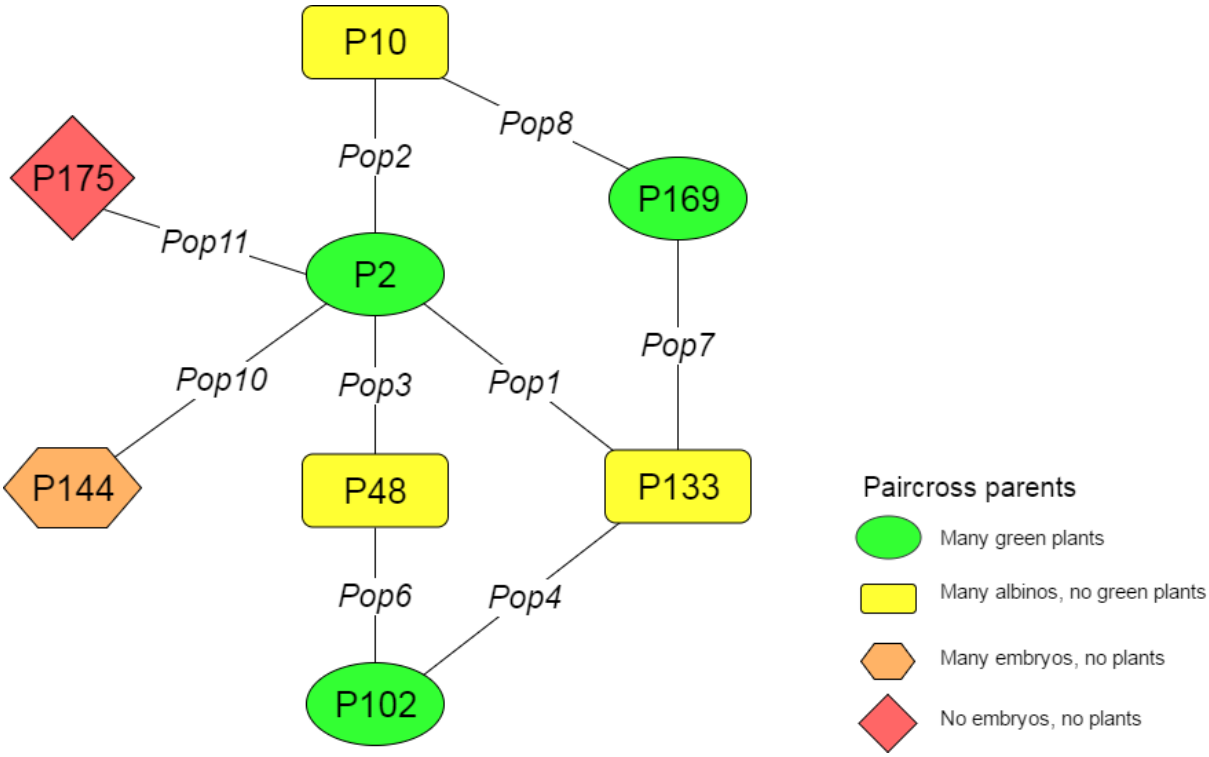

Figure 4.1 Names and anther culture response characteristics of the paircross parents of the populations (Pop) evaluated in this study.

4.2.2 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 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 hours before being used for AC.

After the cold treatment, spikes were surface sterilized for 1 minute in 70% ethanol with a drop of Tween-20, followed by stirring for 10 minutes 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 $Ca(NO_3)_2 \cdot 4\ H_2O$ concentration was increased to 140 mg L^{-1}, the $MnSO_4 \cdot H_2O$ concentration was decreased to 4.9 mg L^{-1}, the $FeSO_4 \cdot 7\ H_2O$ concentration to 13.9 mg L^{-1}, and the $Na_2EDTA \cdot 2\ H_2O$ 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.

4.2.3 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.

4.2.4 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 2017) and using the packages ggplot2 (Wickham 2009) and cowplot (Wilke 2016). Rank-based, non-parametric tests (Kruskall–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).

4.2.5 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.* 2016a), 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 2,500 events were acquired per sample and only measurements with coefficient of variation for G0/G1 peaks <2.5% were accepted.

4.3 Results

4.3.1 Phenotypic responses to anther culture

A total of 351 genotypes, derived from 9 distinct paircross 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 (**Figure 4.2**; **Suppl. Figure S4.1**). 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 (**Figure 4.2B**), while populations 3, 4 and 7 included genotypes producing high numbers of plants (**Figure 4.2C**). Population 6 included genotypes with a relatively high plant regeneration frequency (**Figure 4.2D**) and green plant regeneration per 100 EC (**Figure 4.2F**), as well as the anther donor from which the most green plants per 100 AC were obtained (**Figure 4.2E**). Several genotypes producing over 100 green regenerants per 100 AC could be identified in all populations, except population 11.

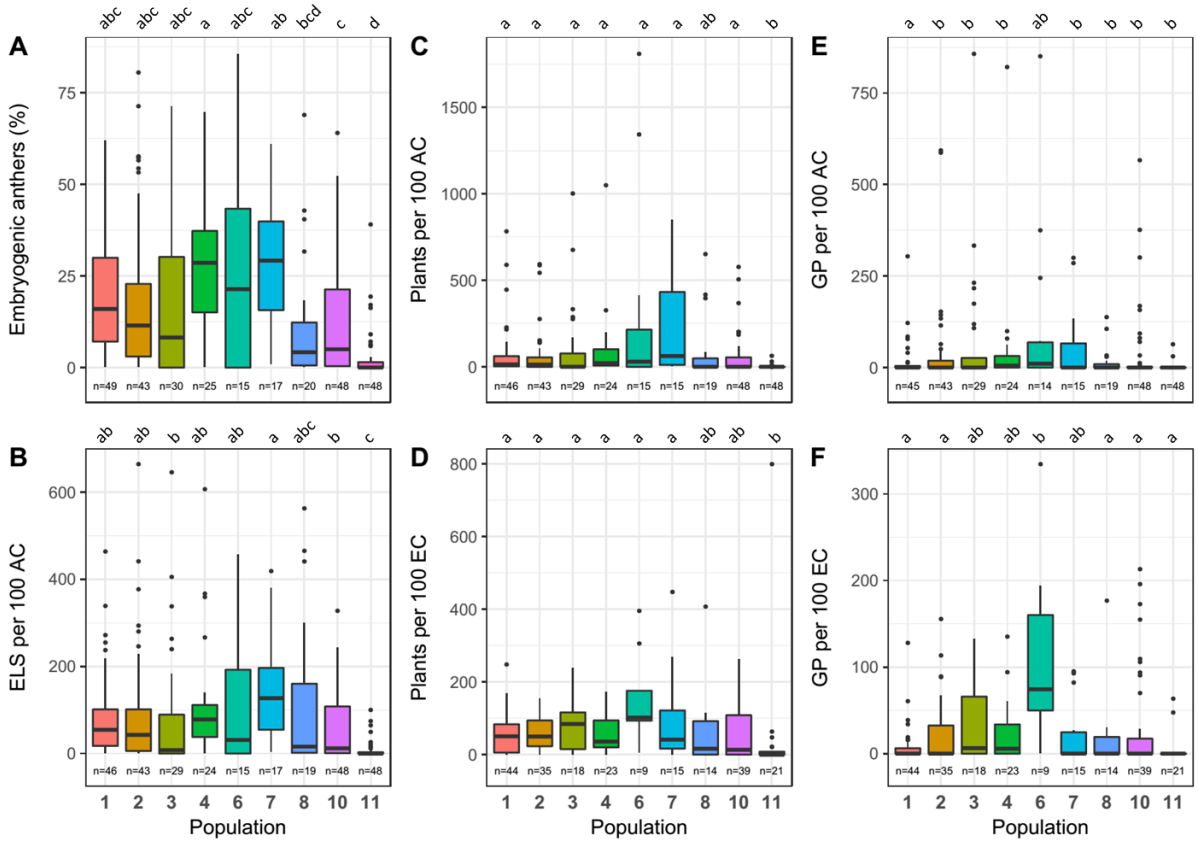

Figure 4.2 Boxplots of the responses of the evaluated populations to anther culture. The letters above each plot denote significant differences between populations ($P \leq 0.05$). **A** – percentage of embryogenic anthers; **B** – number of embryo-like structures (ELS) per 100 anthers cultured (AC); **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**.

Genotypic differences were observed for the percentages of green plants that were regenerated, both within and between the evaluated populations (**Figure 4.3**). 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.

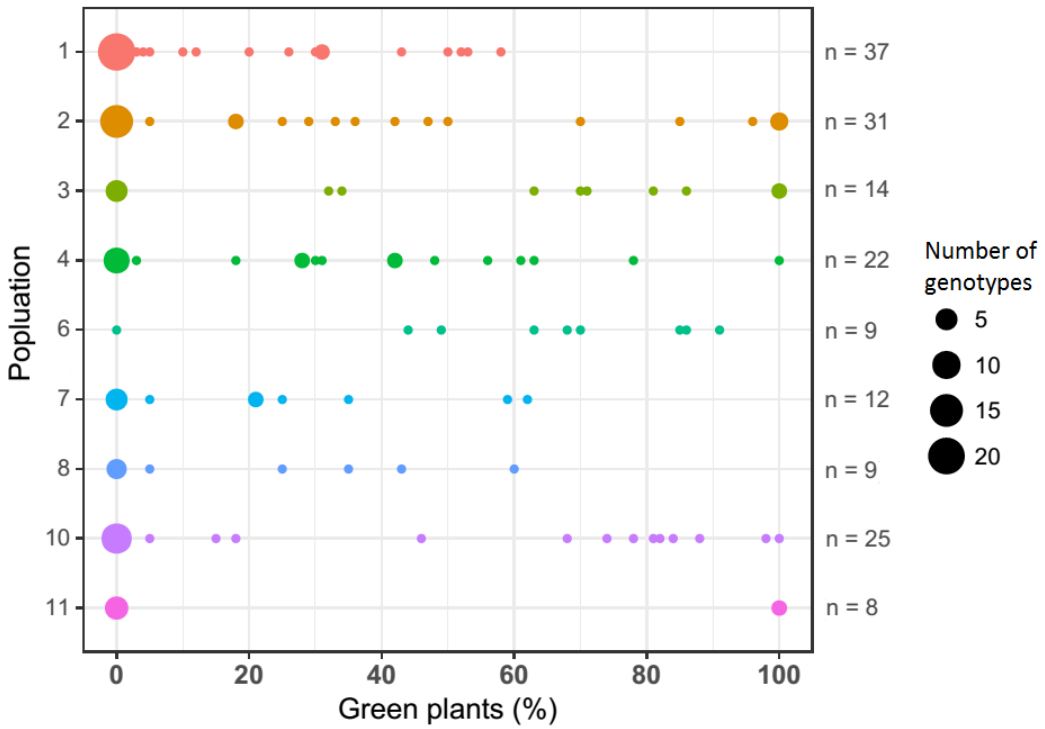

Figure 4.3 Percentages of green plants per genotype per population, regenerated during the 2015 experiments.

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 4.1**). 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.

Table 4.1 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.

4.3.2 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 (**Figure 4.4A-II**). Similarly, anthers of 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 (**Figure 4.4B-I**). The same effect was observed for plants and albino plants per 100 AC ($P \leq 0.05$; **Suppl. Table S4.2**). 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 (**Figure 4.4B-III**; **Suppl. Table S4.1**).

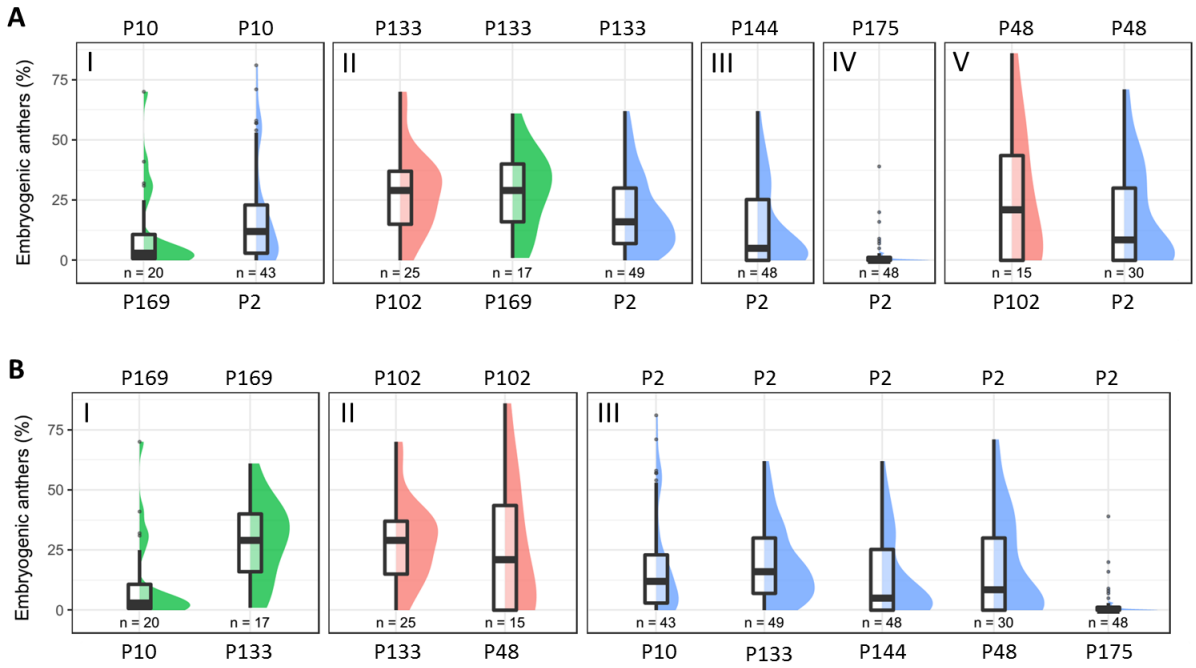

Fig. 4.4 Boxplots with attached density plots of the percentage of embryogenic anthers. **A** – grouped by common non-androgenic paircross parent (P10, P133, P144, P175 and P48); **B** – grouped by common androgenic paircross parent (P169, P102 and P2). Combination P133 and P2 performed significantly 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**).

4.3.3 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 less than 20 plants per 100 AC (n = 102) in order to detect inter-trait relationships relevant in more androgenic material (**Table 4.2**). Many positive and highly significant ($P \leq 0.001$) correlations were found, such as ρ = 0.80 between plants per 100 AC and green plants per 100 AC. Embryo production had a high positive correlation with plant production (ρ = 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 (ρ = 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.

Table 4.2 Ranked correlations (Spearman's ρ) between traits evaluated in 2015, calculated over all populations, excluding genotypes that produced less than 20 plants per 100 anthers cultured (n = 102). 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.

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**

A – albinos; AC – anthers cultured; EA – embryogenic anthers; EC – ELS cultured; ELS – embryo-like structures; GP – green plants; *P* – plants. *** $P \leq 0.001$; ** $P \leq 0.01$; * $P \leq 0.05$; ns – not significant.

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 is rather low in all but population 1 (n = 78). A clear effect of the year was observed and the anther donors performed significantly better in 2015 for almost all traits (**Figure 4.5**). Ranked positive correlations between the years ranged from ρ = 0.45 for plants or green plants per 100 EC ($P \leq 0.001$) to ρ = 0.54 for the two embryo related traits ($P \leq 0.001$).

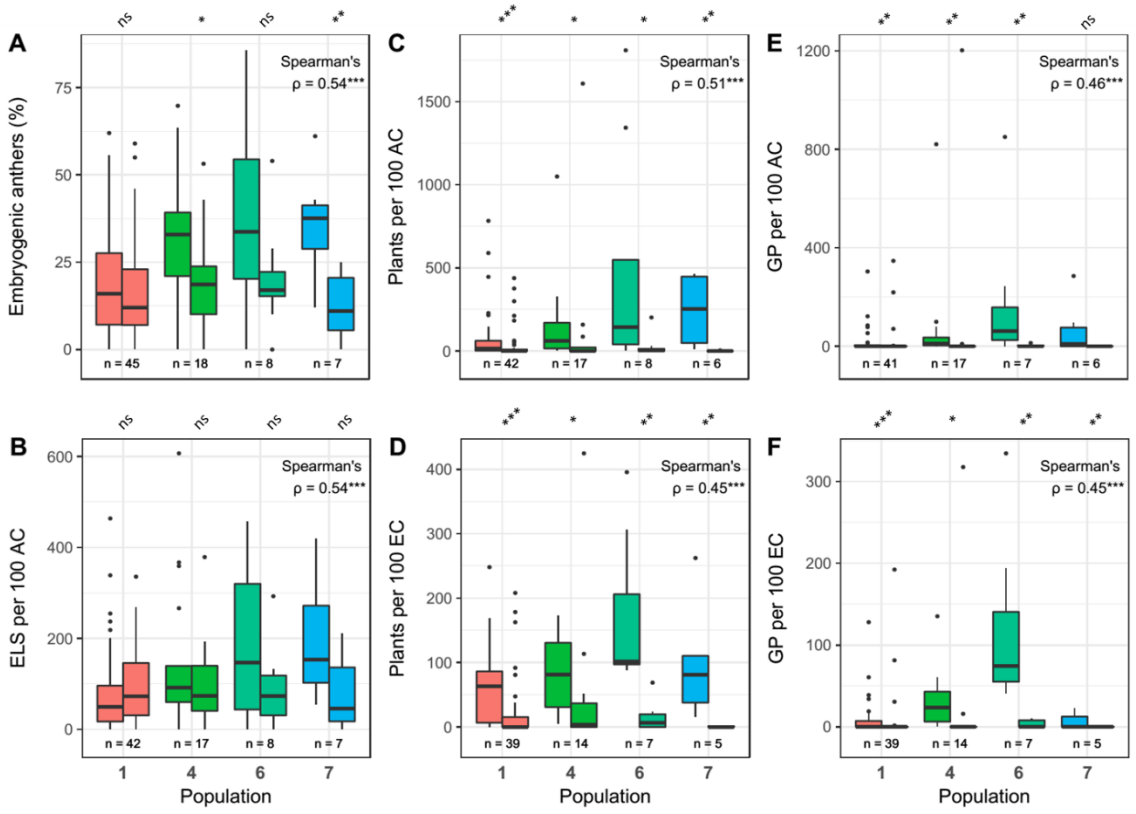

Fig. 4.5 Boxplots of the responses of populations 1, 4, 6 and 7 to anther culture in both 2015 (left) and 2016 (right). Spearman's ρ for the overall correlation between years per trait is given within the plots. Significance levels of the differences between the years for each trait and population are 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.

4.3.4 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 (2*n*), 48 tetraploid (4*n*), and even a few triploid (3*n*), heptaploid (7*n*), and mixoploid (e.g. *n* and 2*n*) 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 0-9 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.

4.4 Discussion

4.4.1 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 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 (Żur *et al.* 2014; Fábián *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 4.1**) 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).

4.4.2 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 paircross 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 (**Figure 4.2**). 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 (Tuvesson *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 (Tuvesson *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 (Tuvesson *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 paircross 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).

4.4.3 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 ρ = 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 (ρ = -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 (ρ = -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.

4.4.4 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 late and erratic flowering caused by incomplete vernalization in 2016 (**Figure 4.5**). 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 ρ = 0.45-0.54 ($P \leq 0.001$) 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).

4.4.5 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 extend inbreeding depression affects their agronomic performance.

4.5 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 programmes in the forage grasses.

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5. Genetic loci governing androgenic capacity in perennial ryegrass (*Lolium perenne* L.)

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Abstract

Immature microspores can be induced to switch developmental pathways from gametogenesis to embryogenesis and subsequently regenerate into homozygous, diploid plants in vitro. Such androgenic production of doubled haploids may be a practically feasible method of inbred line production in self-incompatible species. Therefore, increasing the generally low androgenic capacity of perennial ryegrass (*Lolium perenne* L.) germplasm would enable efficient homozygous line production, so that a more effective exploitation of heterosis through hybrid breeding schemes can be realized. Here, we present the results of a genome-wide association study in a heterozygous, multi-parental perennial ryegrass population (n = 391) segregating for androgenic capacity. Genotyping by sequencing was used to interrogate gene dense genomic regions and revealed over 1100 polymorphic sites. Between 1 and 10 quantitative trait loci (QTL) were identified for anther response, embryo and total plant production, green and albino production and regeneration. Most traits were under polygenic control by several minor QTL, although a major QTL on linkage group 5 was associated with green plant regeneration. Distinct genetic factors seem to affect green and albino plant recovery. Two intriguing candidate genes, encoding chromatin binding domains of the developmental phase transition regulator, Polycomb Repressive Complex 2 (PCR2), were identified. Our results shed the first light on the molecular mechanisms behind perennial ryegrass microspore embryogenesis and enable marker-assisted introgression of androgenic capacity into recalcitrant germplasm of this forage crop of global significance.

Abbreviations

ELS	embryo-like structures
AC	anther(s) culture(d)
EC	ELS cultured
DH	doubled haploid
K-W	Kruskal-Wallis
AP	albino plants
GP	green plants
RA	responsive anthers

5.1 Introduction

In contrast to animals, plant cellular differentiation (cell fate) is both flexible and reversible (Walbot and Evans 2003). In immature male gametophytic cells, a totipotent state can be induced through the application of a stress treatment. Subsequent de-differentiation of such cells into the embryogenic pathway may then be stimulated via their cultivation under suitable in vitro conditions. This process, known as microspore embryogenesis (ME) or androgenesis, ultimately results in the recovery of haploid or, via spontaneous or induced chromosome doubling, diploid completely homozygous individuals (Seguí-Simarro and Nuez 2008). Segregating populations of male gametophytes can thus be transformed into doubled haploids (DHs) in a single generation. These are of great value to fundamental research as well as plant breeding (Forster *et al.* 2007). The practical utility of androgenesis ultimately depends on the efficient production of large numbers of microspore-derived embryos capable of regeneration into green, fertile plants.

The optimum stress and in vitro culture conditions for successful androgenesis are highly species and genotype-dependent (Seguí-Simarro 2010; Dwivedi *et al.* 2015). Through decades of empirical research, highly effective isolated microspore culture (IMC) protocols have been developed for barley (*Hordeum vulgare* L.), rapeseed (*Brassica napus* L.) and tobacco (*Nicotiana* spp.). Unfortunately, many economically (Solanaceae, fruit trees) and academically (*Arabidopsis*) important species remain recalcitrant (Seguí-Simarro 2015). In monocots, and grasses in particular, high rates of albinism further limit androgenic efficiency (Kumari *et al.* 2009). Apart from efforts aimed at establishing which external factors are critical for efficient androgenesis, attempts to uncover the genetic factors controlling ME and plant regeneration have been made.

In many cereal crops, linkage mapping studies have identified chromosomal regions associated with traits related to androgenesis. Quantitative trait loci (QTL) related to embryo production, for example, have been reported in wheat (*Triticum aestivum* L.) (Agache *et al.* 1989), barley (Manninen 2000) and triticale (× *Triticosecale* Wittm.) (González *et al.* 2005; Krzewska *et al.* 2012). The combined effect of two QTL on barley chromosomes 5H and 6H explained 51% of variation in green plant recovery (Chen *et al.* 2007), although only one QTL on chromosome 3H was implicated in a different study (Muñoz-Amatriaín *et al.* 2008). Two regions on wheat chromosomes 1B and 7B explained 53% of the observed variation in albinism (Nielsen *et al.* 2015), QTL for which have also been reported in barley and triticale (Bregitzer and Campbell 2001; Krzewska *et al.* 2015). However, due to a lack of protocol uniformity, the diversity of material under study and the high variability inherent to tissue culture, consensus amongst these types of studies is low (Bolibok and Rakoczy-Trojanowska 2006; Seldimirova and Kruglova 2015). In addition, genes underlying any of the reported QTL have not been identified.

Nevertheless, a number of candidate genes have been associated with high levels of ME and plant regeneration by means of gene expression experiments (reviewed in Hand *et al.* 2016). For example, expression of somatic embryogenesis receptor kinase (SERK) gene *SERK1*, and in some cases *SERK2*, was correlated with embryo production and plant regeneration in species such as *Arabidopsis*, rapeseed, maize (*Zea mays* L.) and wheat (Hu *et al.* 2005; Singla *et al.* 2008; Podio *et al.* 2014; Ahmadi *et al.* 2016; Seifert *et al.* 2016). Overexpression of the *APETALA 2* (*AP2*) transcription factor *BABYBOOM* (*BBM*), *WUSCHEL* (*WUS*) and *AGAMOUS*-like (*AGL*) genes, led to the production of ectopic somatic embryos in *Arabidopsis*, rapeseed and a number of monocot species and improved in vitro regeneration frequencies (Boutilier 2002; Muñoz-Amatriaín *et al.* 2009b; Lowe *et al.* 2016). Other examples of genes that may control ME are the arabinogalactan-related *EARLY CULTURE ABUNDANT 1* (*ECA1*) (Vrinten *et al.* 1999), Polycomb Group (PcG) proteins including *FERTILIZATION INDEPENDENT ENDOSPERM* (*FIE*) (Hand *et al.* 2016), BURP-domain proteins like *BnBNM2* (Boutilier 2002; Tsuwamoto *et al.* 2007; Joosen *et al.* 2007; Malik *et al.* 2007) and the *LEAFY COTYLEDON* (*LEC*) family of

transcription factors (Gruszczyńska and Rakoczy-Trojanowska 2011; Soriano *et al.* 2013; Elahi *et al.* 2016). Again, similar to the linkage mapping studies, the use of different species, treatments and gene expression platforms as well as the complexity of the system under study, prohibit conclusive identification of the genes of greatest importance to successful androgenesis (Soriano *et al.* 2013).

Chromosomal regions or genes associated with androgenic capacity in the most widely grown forage species in temperate agriculture, perennial ryegrass (*Lolium perenne* L.), have not yet been identified. Previous studies concluded that perennial ryegrass' androgenic capacity is under polygenic control, with distinct genetic factors influencing embryo production, plant regeneration and green or albino plant production (Olesen *et al.* 1988; Boppenmeier *et al.* 1989; Opsahl-Ferstad *et al.* 1994b; Madsen *et al.* 1995; Begheyn *et al.* 2017). Additive and dominance effects play a role in embryo and plant production, while green plant production involves dominance effects or the complementation of recessive beneficial alleles. Environmental rather than genetic factors may be the main cause of the high incidence of albinism exhibited by many genotypes (Begheyn *et al.* 2017).

In concert with recent efforts to move towards hybrid perennial ryegrass breeding, the potential of in vitro androgenesis for the efficient production of homozygous lines has been recognized (Arias Aguirre *et al.* 2011; Begheyn *et al.* 2016a; Manzanares *et al.* 2016a; Sykes *et al.* 2016). To overcome the problematic recalcitrance of most breeding germplasm, molecular marker-based introgression of beneficial alleles has been proposed (Halberg *et al.* 1990; Andersen *et al.* 1997). Therefore, the main objective of our study was to identify genetic loci associated with androgenic capacity in a multi-parental perennial ryegrass population via a genome-wide association study (GWAS). In addition, we aimed at identifying potential causal genes that may provide clues to the molecular mechanisms behind ME and plant regeneration in this important member of the grass family.

5.2 Materials and Methods

5.2.1 Plant material and anther culture procedure

A detailed description of most of the plant material and the in vitro AC procedure used here can be found in (Begheyn *et al.* 2017). Briefly, nine perennial ryegrass genotypes with distinct androgenic capacities were paircrossed as part of a DH induction programme at the DLF A/S research station in Store Heddinge, Denmark (**Suppl. Table S5.1**). Eleven populations of paircross offspring were grown in 1 L soil filled pots in an unheated greenhouse in Eschikon, Switzerland, vernalized and used as anther donors in 2015 and 2016. Spikes containing microspores in the late-uninucleate stage were harvested and subjected to a 4°C cold stress treatment of 24-72 hours in the dark. After surface sterilization, anthers were aseptically excised and cultured on an adapted 190-2 induction medium (Wang and Hu 1984) in a 90 mm Petri dish, incubated at 26°C with a 16 h photoperiod. After 6-8 weeks, macroscopic embryo-like structures (ELS) were transferred to the regeneration medium for shoot and root induction.

5.2.2 Phenotypic data collection

To quantify androgenic responses of the anther donor genotypes to in vitro AC, eight phenotypic traits were recorded: (1) anther response as a percentage of anthers producing macroscopic ELS (hereafter 'responding anthers' or RA); (2) embryo production as the number of ELS per 100 anthers cultured (AC); (3) plant, (4) green plant and (5) albino plant production, recorded per 100 AC; and (6) plant, (7) green plant and (8) albino plant regeneration, recorded per 100 ELS cultured. In 2015, a total of 313 genotypes were investigated, while incomplete vernalization prior to 2016 resulted in 116 studied genotypes. A total of 78 genotypes were phenotyped in both years (**Suppl. Table S5.1**; (Begheyn *et al.* 2017)).

5.2.3 DNA extraction

Fresh leaf tissue of the anther donor plants was harvested for DNA extraction on a 96-well plate KingFisher Flex Purification System with KingFisher Pure DNA Plant Kits (Thermo Fisher Scientific, Waltham, MA, USA). Genomic DNA was visualized on a 1% agarose gel and quantified with a NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

5.2.4 Genotyping by sequencing library preparation

Genotyping by sequencing (GBS) libraries were prepared by multiplexing single restriction enzyme digested genomic DNA using 192 unique 5-10 bp barcodes (**Suppl. Table S5.2**), designed with the Deena Bioinformatics online GBS Barcode Generator (http://www.deenabio.com/nl/services/gbs-adapters) and synthesized by Microsynth (Balgach, Switzerland)[1].

Per sample, a 20 µL PstI digestion mixture was prepared, containing 10 µL DNA sample (10 ng μL^{-1}), 1 µL PstI (3.5 U μL^{-1}), 2.5 µL barcoded adaptors (0.1 ng μL^{-1}), 2.5 µL common adaptors (0.1 ng μL^{-1}), 2 µL O buffer and 2 µL H_2O. Samples were digested for 2 h at 37°C. Ligation with T4 ligase, pooling of 96 samples and purification (Qiagen MinElute PCR Purification Kit; Qiagen, Hilden, Germany) were performed according to Elshire *et al.* (2011). Fragments were amplified in volumes of 50 µL, containing 5 µL DNA library, 0.25 µL DreamTaq DNA Polymerase (5 U μL^{-1}), 5 µL 10× DreamTaq Buffer, 5 µL dNTPS (2 mM), 1 µL primers (10 µM; Suppl. Table S2) and 33.75 µL H_2O. Thermocycler steps were as follows: 72°C for 5 min, 95°C for 30 s, 21 cycles of 95°C for 10 s, 65°C for 30 s and 72°C for 30 s, with a 5 min final extension at 72°C (GeneAMP PCR System 9700; Thermo Fisher Scientific, Waltham, MA). All enzymes and their associated buffers were purchased from Thermo Fisher Scientific. Purified (as above) fragments were visualized on a 2200 TapeStation (Agilent Technologies, Santa Clara, CA, USA) to check for presence of adapter dimers and confirm a majority fragment length of 200-400 bp. If adapter dimers were present, an Agencourt AMPure XP bead purification (Beckman Coulter Inc., Brea, CA, USA) was performed.

5.2.5 GBS library sequencing

Two 192-plex and one 39-plex anther donor GBS libraries (423 genotypes in total) were sequenced using 126 bp single-end reads on three lanes of an Illumina HiSeq 2500 platform at the Functional Genomics Center Zurich (FGCZ) of the ETH Zurich, Switzerland.

5.2.6 GBS data processing, read mapping and variant calling

Reads were de-multiplexed using sabre (https://github.com/najoshi/sabre) allowing one mismatch. Using Bash commands and custom Perl scripts, reads were trimmed to 100 bp and the frequency (counts) of unique sequences (tags) was summarized per paircross population. Unique tags were back-transformed to FASTQ format. Bowtie v0.12.7 (Langmead *et al.* 2009) with "--best --strata" and a maximum of two alignments "-m 2" was used to map the FASTQ files to the perennial ryegrass genome v1.0 (Byrne *et al.* 2015). Unmapped tags were filtered out using a custom Perl script, resulting in 141,775,689 (20.2% of de-multiplexed) mapped tags. The SAM files as well as the count files were further processed in R v3.3.3 (R Core Team 2017).

Numerical factors were set to constrain genotyping to reflect the ploidy level of the genotypes (2*n*) and the maximum allele number (four) for paircross populations. Cut off values of 100 for the minor allele frequency (minAF) and eight for the minimum allele count (minAC) were used. Unique position

[1] Keygene N.V. owns patents and patent applications protecting its Sequence Based Genotyping technologies (Truong *et al.* 2012).

identifiers (Upos) were extracted from the SAM files by concatenating the direction (Flag), location (Ref) and position (Pos) data. Low coverage sites were eliminated by retaining only Upos with at least one tag greater than the minAF. From the resulting tags, only those occurring at a frequency greater than 5% were retained.

For genotype calling, all informative, polymorphic nucleotide sites (Isites) across the tags were identified and only informative tags (Itags) with Isites were retained. Two unique alleles at one Isite position were called as heterozygous, while the occurrence of a single allele at one Isite was called as homozygous if its count was greater than the minAC. Informative tags were excluded if the number of unique Isites was greater than the ploidy level, or if the allele number within an Isites was greater than the maximum allele number. Haplotypes were obtained by concatenating alleles at the Isites within each tag, if applicable.

5.2.7 Genome-wide association mapping (GWAS)

Population structure was investigated using STRUCTURE v2.3.4 (Hubisz *et al.* 2009), GAPIT v2 (Lipka *et al.* 2012) as well as the hierarchical clustering hclust() (method = "ward.D") and principal component analysis (PCA) prcomp() functions in R.

Itags were filtered using a minAF threshold of 10% and a minimum of 100 and 50 genotypes in 2015 and 2016, respectively (**Suppl. Figure S5.1**). Since the phenotypic data did not, and could not be made to fit the criteria for parametric testing (Begheyn *et al.* 2017), the non-parametric, rank-based Kruskal-Wallis (K-W) test was used to detect associations between each segregating haplotype (Itag) and the phenotypic traits (Kiviharju *et al.* 2004; Krzewska *et al.* 2012). For each of these K-W tests, 10,000 permutations of the phenotypes were run. Associations were considered significant at a K-W LOD of 3.0 or higher and a permutation test threshold of 1%. Bonferroni corrected Dunn's tests ($P \leq 0.05$) were carried out *post hoc* to compare haplotypes' trait values. All statistical analyses were performed using custom scripts in Rstudio v1.0.143 (RStudio Team 2015), running R v3.3.3 (R Core Team 2017). The R packages ggplot2 (Wickham 2009) and UpSetR (Lex *et al.* 2014) were used to generate the figures.

Scaffolds of the perennial ryegrass genome v1.0 (Byrne *et al.* 2015) containing significant Itags will hereafter be referred to as "significant scaffolds".

5.2.8 Positioning the significant scaffolds on the GenomeZipper

Significant scaffolds were compared against the genome sequences of *Brachypodium distachyon*, rice (*Oryza sativa* Japonica Group) and sorghum (*Sorghum bicolor* L.) using a BLASTN search ($E \leq 1e^{-5}$, sequence identity ≥ 85%, match length of ≥ 150 bp). Matches were compared to the perennial ryegrass GenomeZipper (Pfeifer *et al.* 2013) in order to obtain the (approximate) locations of the scaffolds of interest on the linkage groups (LGs).

5.2.9 Gene annotation

Gene prediction and annotation has been performed as described in Knorst *et al.* 2017 (*under revision*; annotation data were deposited at zenodo.org).

5.2.10 Data availability

Sequences will be made available upon acceptance.

5.3 Results

5.3.1 Phenotypic data

The genotype-dependent response to AC, the wide segregation of androgenic capacity within and the differences between the performance of the bi-parental mapping populations, have been

described in detail in Begheyn *et al.* (2017). In addition, a further eighteen genotypes were included in this study (populations 12 and 15; **Suppl. Table S5.1**). A detailed summary of the phenotypic traits can be found in **Table 5.1**. A total of 313 and 116 genotypes were subjected to in vitro AC in 2015 and 2016, respectively, with an overlap of 78 genotypes between the two years (Begheyn *et al.* 2017). While observations ranged from zero to several hundred or even over 1,000 in the case of plant and green plant production, the majority were zeros (mode = 0) or close to zero (medians; **Table 5.1**). As a consequence, all of the eight androgenic capacity-related traits were, even upon transformation, not normally distributed (Begheyn *et al.* 2017), which necessitated the use of nonparametric statistics for the GWAS analyses (Rebaï 1997).

Table 5.1 Summary of the androgenic capacity-related phenotypic traits under study (Begheyn *et al.* 2017).

Trait	Min	Max	Median	Interquartile range	Number of genotypes
2015					
RA (%)	0	86	7.9	27.5	313
ELS per 100 AC	0	665	21	94.9	307
Plants per 100 AC	0	1810	2.4	54	305
Plants per 100 EC	0	800	38.5	95.2	229
GP per 100 AC	0	1530	0	6	297
GP per 100 EC	0	335	0	25	229
AP per 100 AC	0	705	2	28	297
AP per 100 EC	0	800	21.1	52.6	229
2016					
RA (%)	0	87	13	18	116
ELS per 100 AC	0	933	73	117	116
Plants per 100 AC	0	1609	0	9	116
Plants per 100 EC	0	425	0	18.3	105
GP per 100 AC	0	1203	0	0	115
GP per 100 EC	0	318	0	0	104
AP per 100 AC	0	942	0	6.6	115
AP per 100 EC	0	270	0	14.4	104

AC – anthers cultured; AP – albino plants; ELS – embryo-like structures; EC – 100 ELS cultured; GP – green plants; RA – responsive anthers

5.3.2 Genotyping by sequencing (GBS)

Sequencing of the GBS libraries yielded a total of 884,174,849 raw, or 701,662,007 de-multiplexed reads. Of these, 141,775,689 (20.2%) were mapped to the perennial ryegrass genome assembly v1.0 (Byrne *et al.* 2015). After removing non-polymorphic tags (75.6%) and stringent filtering (see Materials and Methods), 1120 and 1079 informative tags of 100 bp, containing a polymorphic SNP or haplotype, could be used for the analysis of the 2015 and 2016 datasets, respectively (**Suppl. Figure S5.1**). While the majority contained a single SNP, 25.8% (2015) and 24.2% (2016) of informative tags harboured two or more SNPs. Such sets of SNPs on single tags were treated as haplotypes in subsequent analyses.

Given the multi-parental pedigree of the genotypes used in this study, the necessity for applying a correction for population stratification or structure (kinship) was investigated. No evidence for either was found upon analysis of the genotypic data using STRUCTURE (Porras-Hurtado *et al.* 2013), a kinship matrix (VanRaden 2008) or hierarchical clustering. In addition, the two principal components of the

PCA explained 76.3% and 10.4% of variation, respectively (**Suppl. Figure S5.2**). It was therefore not deemed necessary to include population structure or relatedness corrections in subsequent analyses.

5.3.3 Genome-wide association study (GWAS)

Analysis of the 2015 dataset resulted in the identification of significant associations (LOD ≥ 3.0) between six of the studied traits and nine SNPs as well as five haplotypes. Because two of the tags harbouring these polymorphisms mapped back to the same scaffold (2554) of the perennial ryegrass genome assembly (Byrne *et al.* 2015), a total of thirteen significant scaffolds were identified (**Table 5.2**). No significant associations were found for plant or albino plant regeneration. Analysis of the smaller 2016 dataset yielded seven significant scaffolds (LOD ≥ 3.0) for six traits (**Table 5.2**). No significant associations were found for plant production and regeneration and none of the scaffold was significantly associated with a trait in both years given the 3.0 LOD threshold.

Since non-parametric testing does not allow for an estimation of QTL or allelic effects, allele or haplotype medians per significant scaffold and trait, combined with Dunn's tests *post hoc* to ascertain significant differences ($P \leq 0.05$), are presented instead (**Table 5.2**). In the 2015 dataset, for example, differences between the medians of the most and least beneficial SNP or haplotype ranged from 9.7 to 18.1 for percentage responsive anthers, 31.5 to 54.2 ELS per 100 AC and 4.9 to 27 plants per 100 AC. The 2016 dataset included a haplotype (TTTC/TTTC) associated with a median albino plant regeneration of 37.5 compared to 0 for the other haplotypes (CCCG/TTTC and CCCG/CCCG) of the same significant scaffold (3194). The smallest significant differences in median, of less than 1 and 1.2 in the 2015 and 2016 datasets, respectively, were observed for green plant production. Nevertheless, for green plant regeneration, the beneficial allele on scaffold 3723 was associated with a median increase of 62.2 green plants per 100 EC compared to the least beneficial allele (**Table 5.2**).

Table 5.2 Overview of the significant scaffolds of the perennial ryegrass genome assembly (Byrne *et al.* 2015) detected for each trait (LOD ≥ 3.0). Significant differences ($P \leq 0.05$) between phenotypic medians are indicated with letters.

Trait	Scaffold	LG	Position (cM)	LOD	Allele or haplotype	Median	Allele or haplotype	Median	Allele or haplotype	Median
2015										
RA (%)	815	1	33.0-33.3	3.0	C/C	21.0 [a]	C/T	6.9 [b]	T/T	6.1 [b]
	233	4	40.4-40.5	3.9	AC/AC	17.1 [a]	AC/GT	10.8 [a]	GT/GT	1.3 [b]
	16597	4	52.3-52.4	3.4	GAG/GAG	19.6 [a]	CGA/CGA	5.2 [b]	CGA/GAG	1.5 [b]
	1669	5	0	3.2	G/G	14.7 [a]	G/T	1.3 [b]		
	2554_2	5	28.5	3.8	C/C	11.7 [a]	C/T	2.0 [b]		
	2075	7	43.6-43.7	3.3	GT/GT	19.4 [a]	TC/TC	13.8 [a]	GT/TC	1.3 [b]
	4385	7	46.5	3.1	TG/TG	19.0 [a]	GA/TG	14.2 [a]	GA/GA	2.4 [b]
ELS/100AC	815	1	33.0-33.3	3.4	C/C	73.6 [a]	C/T	13.1 [b]	T/T	21.6 [b]
	233	4	40.4-40.5	3.1	AC/AC	55.9 [a]	AC/GT	36.9 [ab]	GT/GT	1.7 [b]
	16597	4	52.3-52.4	3.9	GAG/GAG	62 [a]	CGA/CGA	8.3 [b]	CGA/GAG	2.4 [b]
	1669	5	0	3.4	G/G	41.9 [a]	G/T	0.7 [b]		
	2554_2	5	28.5	4.5	C/C	34.9 [a]	C/T	3.4 [b]		
	4385	7	46.5	3.9	TG/TG	54.6 [a]	GA/TG	32.8 [a]	GA/GA	0.8 [b]
	10161	-	-	3.5	C/T	49.7 [a]	T/T	47 [a]	C/C	5.0 [b]
Plants/100 AC	16597	4	52.3-52.4	3.0	GAG/GAG	27.0 [a]	CGA/CGA	0.0 [b]	CGA/GAG	0.0 [b]
	2554_2	5	28.5	4.8	C/C	4.9 [a]	C/T	0.0 [b]		

Trait	Scaffold	LG	Position (cM)	LOD	Allele or haplotype	Median	Allele or haplotype	Median	Allele or haplotype	Median
	10161	-	-	3.3	C/T	7.9 [a]	T/T	3.8 [a]	C/C	0.0 [b]
GP/100AC	6436	2	79.6-79.8	3.1	T/T	1[a]	C/T	0.0 [b]	C/C	0.0 [a]
GP/100EC	3723	5	4.5-25.4	3.1	C/C	64.2 [a]	C/T	2.0 [a]	T/T	0.0 [b]
AP/100AC	16597	4	52.3-52.4	3.2	GAG/GAG	16.3 [a]	CGA/CGA	0.0 [b]	CGA/GAG	0.0 [b]
	2554_1	5	28.5	4.0	G/G	5.8 [a]	A/G	0.0 [b]		
	2554_2	5	28.5	5.3	C/C	4.0 [a]	C/T	0.0 [b]		
	6186	7	43.6-43.7	3.2	CA/CA	12.7 [a]	GT/GT	9.7 [a]	CA/GT	0.0 [b]
	1607	7	51.6-51.7	3.0	A/A	13.1 [a]	A/C	6.1 [a]	C/C	0.0 [b]
	123	7	62.4-62.8	3.3	G/G	13.8 [a]	A/G	0.0 [b]	A/A	0.0 [b]
2016										
RA (%)	8920	4	22.2-22.3	3.4	CC/TT	21.0 [a]	TT/TT	13.0 [a]	CC/CC	9.0 [b]
	15142	5	28.2	3.2	A/G	22.0 [a]	A/A	11.0 [ab]	G/G	8.0 [b]
	60	5	28.5	3.3	T/T	34.0 [a]	C/T	11.0 [b]	C/C	8.5 [b]
ELS/100AC	8920	4	22.2-22.3	3.3	CC/TT	102.0 [a]	TT/TT	89.0 [a]	CC/CC	28.5 [b]
	813	5	28.5-28.5	3.2	A/G	173.0 [a]	G/G	36.0 [b]		
GP/100AC	127	1	56.1-57.5	3.9	G/G	1.2 [a]	A/G	0.0 [b]	A/A	0.0 [b]
GP/100EC	127	1	56.1-57.5	4.1	G/G	1.6 [a]	A/G	0.0 [b]	A/A	0.0 [b]
AP/100AC	7045	7	37.5-38.6	3.3	C/C	21.1 [a]	C/T	0.0 [b]	T/T	0.0 [b]
AP/100EC	3194	1	30.9-31.1	3.0	TTTC/TTTC	37.5 [a]	CCCG/TTTC	0.0 [b]	CCCG/CCCG	0.0 [b]
	7045	7	37.5-38.6	3.0	C/C	19.8 [a]	C/T	0.0 [b]	T/T	0.0 [b]

AP – albino plants; AC – anthers cultured; ELS – embryo-like structures; EC – ELS cultured; GP – green plants; LG – linkage groups; RA – responsive anthers.

Most significant associations were found for the percentage of responsive anthers (ten associations), embryo production (nine) and albino plant production (seven; **Figure 5.2**). Using the 2015 dataset, four scaffolds (815, 233, 1669 and 4385) were significant for both the percentage of responsive anthers as well as ELS production, while two scaffolds (16597 and 2554) were significantly associated with percentage responsive anthers and the production of ELS, plants and albino plants. Scaffold 10616 was significantly associated with ELS and plant production. Three scaffolds, 8920, 127 and 7045 were found to be significant for two traits using the 2016 dataset.

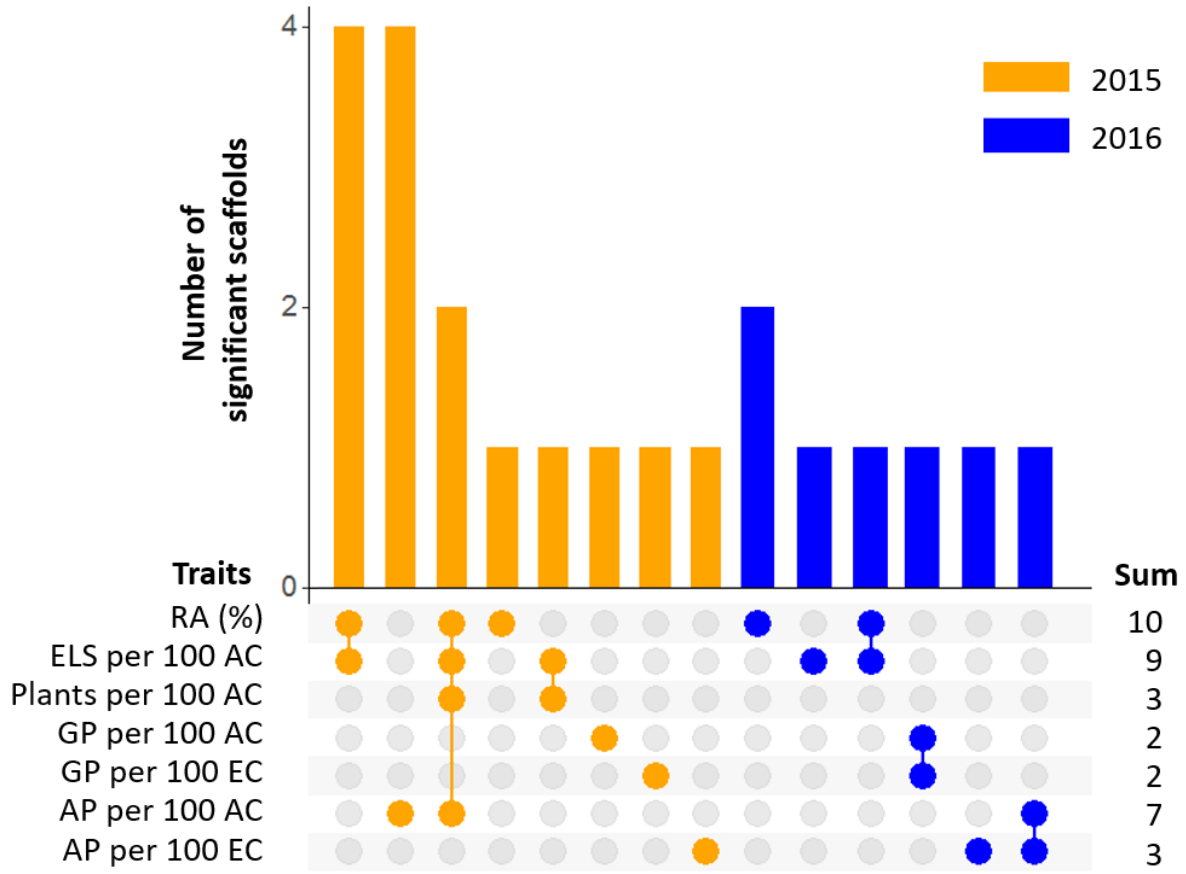

Figure 5.2 Overview of the number of significant scaffolds per trait or, shown with connected dots, per group of traits (*bars*) and the total number of significant scaffolds per trait (*sum*). AP – albino plants; AC – anthers cultured; ELS – embryo-like structures; EC – ELS cultured; GP – green plants; RA – responsive anthers.

5.3.4 Positioning significant scaffolds on the GenomeZipper

By comparing *B. distachyon*, rice and sorghum gene homologs identified on the significant scaffolds with those anchored on the perennial ryegrass GenomeZipper (Pfeifer *et al.* 2013), all but one scaffold could be assigned approximate positions on the LGs (**Figure 5.3**). Even so, confidence in the positioning varied from case to case. For example, the approximate positions of scaffolds 123, 127, 233, 813, 2075, 3194, 3723, 6186, 15142 and 16597 were resolved via one or several exact gene matches to the same location on the GenomeZipper. Scaffolds 60, 815, 1607, 1669, 2554, 4385, 6436, 7045 and 8920 were positioned (approximately) using three to ten genes that were not anchored on the GenomeZipper, but could be placed between several genes anchored at the same location. Scaffold 10616 could not be assigned a location because no significant BLASTN hits of sufficient length were obtained.

Even though no scaffold was found to be significant in both years, scaffolds identified in different years were positioned in similar locations on the GenomeZipper LGs (**Figure 5.3**). Scaffolds 815 (2015) and 3194 (2016) are approximately 2 cM apart on LG 1 for example, while scaffolds 60, 813 and 15142 (2016) and 2554 (2015) are all positioned within a 0.3 cM region on LG 5. On the lower middle region of LG 7, scaffolds 2075 and 6186 (43.6 to 43.7 cM) and 4383 (46.5 cM) from the 2015 dataset were positioned in close proximity to each other.

No scaffolds were positioned on LGs 3 and 6. Scaffolds associated with the percentage of responsive anthers, ELS production and at least one of the albino plant-related traits were positioned on LGs 1, 4, 5 and 7, mostly relatively close together. Also amidst these, on LGs 4 and 5, were the two plant

production-related scaffolds (2554 and 16597) that could be placed on the GenomeZipper. The three scaffolds (127, 3723 and 6463) significantly associated to the green plant-related traits were some distance away from the scaffolds associated to the other traits. In fact, scaffold 6436 was the only scaffold positioned on LG 2.

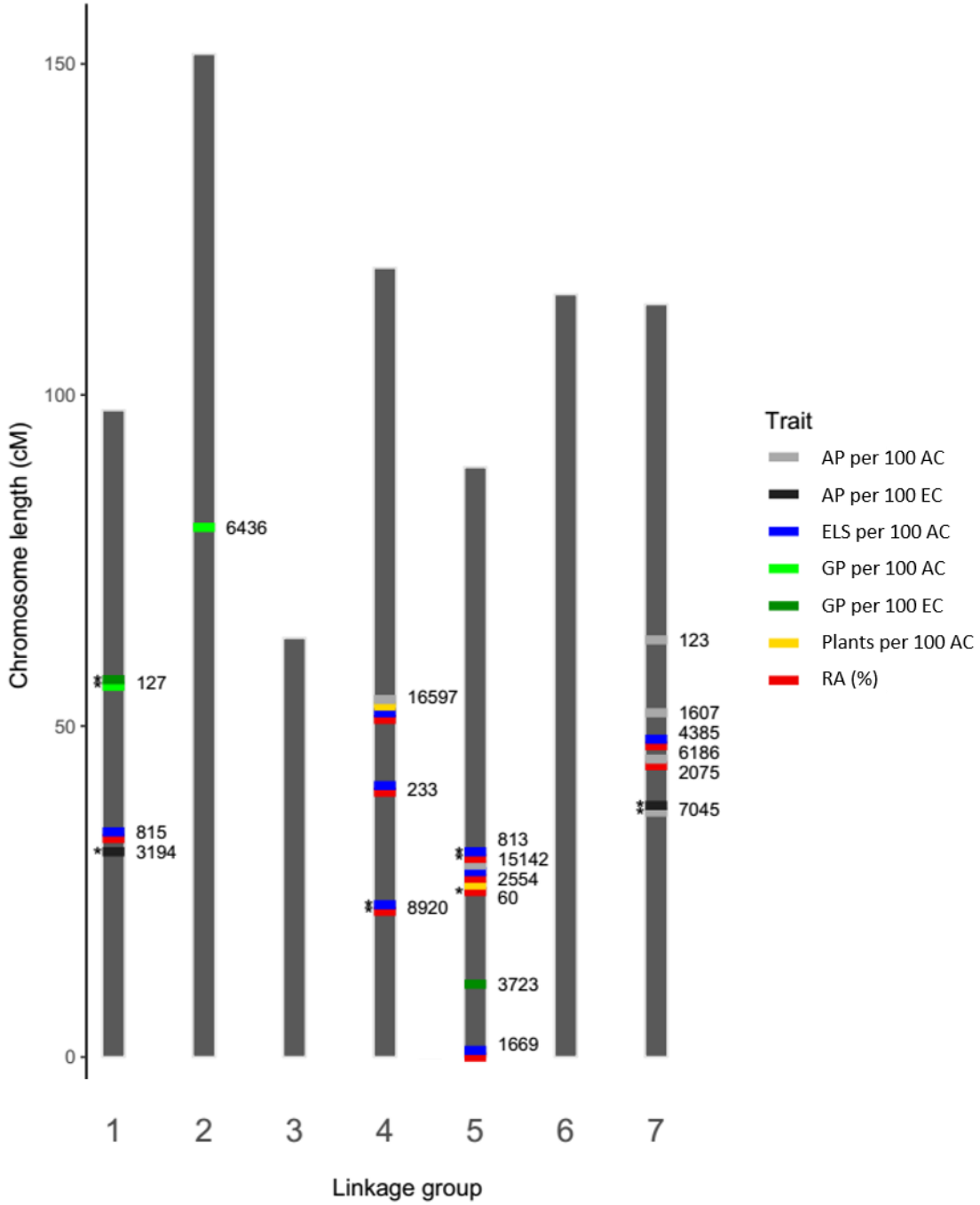

Figure 5.3 Positions of the significant scaffolds detected in 2015 and 2016 (*) on the perennial ryegrass genome as inferred by the perennial ryegrass GenomeZipper (Pfeifer *et al.* 2013). AP – albino plants; AC – anthers cultured; ELS – embryo-like structures; EC – ELS cultured; GP – green plants; RA – responsive anthers.

5.3.5 Gene annotations

Between one and four predicted genes were annotated for each significant scaffold, with the exception of scaffold 10616 (**Suppl. Table S5.2**). On scaffold 1607 for example, sequence homology to

the Arabidopsis *SERRATE* (*SE*) gene was found, while homologues of two domains of Polycomb Repressive Complex 2 (PRC2), *FERTILIZATION-INDEPENDENT ENDOSPERM* (*FIE*) and *CURLY LEAF* (*CLF*) were identified on scaffolds 4383 and 7045, respectively.

5.4 Discussion

Here, we present the first report of genetic loci associated with in vitro androgenesis in perennial ryegrass. Between two and ten QTL (LOD ≥ 3.0) for anther response percentage, embryo production, total plant production as well as green and albino plant production and regeneration were identified on five of the seven perennial ryegrass LGs. Additionally, several intriguing candidate genes that may be responsible for the observed phenotypic differences were predicted on the QTL-harbouring scaffolds of the perennial ryegrass genome assembly (Byrne *et al.* 2015). These results enable the development of the first molecular markers for androgenic capacity in perennial ryegrass, from the identified, polymorphic GBS tags. Their availability will help to realize the long-standing aim of efficient, marker-assisted introgression of good responses to in vitro DH induction into recalcitrant germplasm (Halberg *et al.* 1990; Andersen *et al.* 1997).

5.4.1 Multi-parental population GWAS in perennial ryegrass

Contrary to previous QTL studies on androgenic capacity, which were based on linkage mapping in bi-parental populations of up to 100 individuals (Muñoz-Amatriaín *et al.* 2008; Krzewska *et al.* 2012; Nielsen *et al.* 2015), an association mapping approach in a multi-parental population, composed of 391 heterozygous individuals, was applied here. This design increased the presence of distinct alleles, confirmed by the observed phenotypic variation (Begheyn *et al.* 2017), and, due to the recombination between the nine heterozygous parents, ensured high levels of allelic diversity as well as good mapping resolution (Klasen *et al.* 2012; Giraud *et al.* 2014; Wang *et al.* 2017). Around 1100 polymorphic SNPs and haplotypes, identified using a methylation-sensitive GBS protocol, allowed for the genome-wide interrogation of gene-dense regions within the multi-parental mapping population (Byrne *et al.* 2013). Significant population structure was absent, due to the common breeding history of the parental plants used to design the mapping population. This powerful experimental design, combined with robust, non-parametric (K-W) single SNP/haplotype genome-wide analysis and permutation-based validation, was successfully used to detect significant QTL (LOD ≥ 3.0) associated with the component traits of the androgenic response of perennial ryegrass.

5.4.2 A putative major QTL for green plant regeneration on perennial ryegrass LG 5

Authors have often commented on the difficulty of comparing tissue culture experiments, due to highly genotype-specific responses as well as crucial differences in execution and data collection (Bolibok and Rakoczy-Trojanowska 2006; Seldimirova and Kruglova 2015). Fortunately, comparative genomics studies within the grass family allow for an interspecific comparison of cereal AC and IMC QTL studies, albeit at the chromosomal level (Devos 2005). Most homologous grass chromosomes have been associated with all of the androgenicity-related traits at least once, however, and a common pattern is not obvious. One possible exception is a putative locus controlling green plant regeneration, which was identified on Triticeae chromosome group 5 and reported to affect 12-37% of the phenotypic variation in barley, rice (chromosome 9), triticale and wheat (He *et al.* 1998; Torp *et al.* 2001; Chen *et al.* 2007; Muñoz-Amatriaín *et al.* 2008; Krzewska *et al.* 2012). Intriguingly, we identified a putative major QTL, associated with a median increase of 62 green plants per 100 AC, on perennial ryegrass LG 5 as well (Pfeifer *et al.* 2013). This locus is therefore of great interest and its further investigation, for example using fine-mapping approaches, may lead to the identification of the gene with a considerable effect on green plant regeneration in the grass family.

5.4.3 Genetic control of androgenic capacity

A relatively large number of QTL with modest effects were associated with androgenic traits, such as anther response percentage (ten QTL), embryo production (nine QTL) and albino plant production (seven QTL). In addition, many QTL were shown to affect several traits, confirming the high correlations between, for example, embryo production and anther response as well as plant production observed earlier (Begheyn *et al.* 2017). Similar results have been reported by other groups (Murigneux *et al.* 1994; Beaumont *et al.* 1995; Manninen 2000; Krzewska *et al.* 2012). Finally, QTL detected in 2015 were not detected in 2016 and vice versa, although the QTL identified on scaffold 2075 using the 2015 dataset had a LOD of 2.0 using the 2016 dataset for percentage responsive anthers (results not shown). The discrepancy is probably caused by the fact that only 78 genotypes from four bi-parental crosses were subjected to AC in both years and just 45 of those had the same paircross parents (population 1). Allele frequencies of QTL detected using the 2015 dataset were likely too low, or entirely absent, from the 2016 dataset, which in turn harboured distinct beneficial alleles at a high enough frequency for QTL detection. Although a smaller dataset was used in 2016, several QTL of particular interest were detected. For example, a QTL on scaffold 813 was associated with a major median increase in embryo production of 137 ELS per 100 anthers cultured. In addition, the only QTL (on scaffolds 3194 and 7045) associated with albino plant regeneration, connected with an median increase of 19.8 and 37.5 albino plants per 100 ELS cultured, were detected using this dataset.

All of the above findings may be explained by the fact that both ME and albinism during in vitro culture are under complex, polygenic and heterogeneous control (Seguí-Simarro and Nuez 2008; Makowska and Oleszczuk 2014). A single genetic master switch for ME has never been identified and albino phenotypes can be caused by mutations in as many as 300 nuclear genes (Kumari *et al.* 2009; Hand *et al.* 2016). A significant increase in embryo production may, therefore, be accomplished via the stacking of several genetic loci with modest effect within single genotypes (Madsen *et al.* 1995; Andersen *et al.* 1997; Marhic *et al.* 1998). The production of albinos may be reduced by similar means.

A relatively small number of QTL were associated with plant production, green plant production and green and albino plant regeneration. The three QTL detected for total plant production also affected either embryo production, albino production or both. Conversely, the QTL that influenced green plant production (2 QTL) and regeneration (2 QTL) were not associated with any other traits and positioned at distinct locations on the perennial ryegrass LGs. In addition, only one of the two QTL related to albino plant regeneration affected a second trait, albino plant production. These results do not only confirm the separate genetic control of green and albino plant production capacity reported previously (He *et al.* 1998; González *et al.* 2005; Krzewska *et al.* 2015; Begheyn *et al.* 2017). They also suggest that total plant production and total plant regeneration, for which no QTL were identified at all, may not be of great use to describe androgenic ability. The three phases of in vitro androgenesis that are commonly distinguished, 1) embryo production, 2) plant regeneration and 3) green plant recovery, can, at least in the grass family, be redefined as 1) embryo production, 2a) green plant recovery and 2b) albino plant recovery. Green plant recovery seems to be controlled by fewer loci than albino plant recovery, although environmental influence on albinism may have masked both green plant production and regeneration capacity as well as the QTL associated with them (Begheyn *et al.* 2017).

5.4.4 Candidate genes involved in androgenic response

While the putative function of most candidate genes underlying the QTL identified here has yet to be resolved, several have previously been associated with the regulation of stress response, cell fate change, embryogenesis or organogenesis. The *ISOPRENYLCYSTEINE METHEYLESTERASE-LIKE* 2 (*ICME-LIKE2*) gene annotated on scaffold 123, for instance, is involved in abscisic (ABA) mediated stress

signaling and specifically expressed in reproductive organs of *Arabidopsis* (Lan *et al.* 2010). Similarly, the *VIP HOMOLOG 1* (*VIH1*) gene, identified on scaffold 233, is crucial to certain aspects of jasmonate mediated stress signalling and is mainly expressed in *Arabidopsis* pollen (Laha *et al.* 2015). Phytohormones like ABA and jasmonic acid (JA) have, in fact, been shown to play important roles during androgenesis by ensuring microspore viability through the regulation of stress responses as well as inducing ME via signalling cascades that activate specific gene expression programs (Maraschin *et al.* 2005; Ahmadi *et al.* 2014; Żur *et al.* 2015a). The *Arabidopsis SERRATE* (*SE*) gene, which is involved in chromatin modification and microRNA-mediated gene expression regulation during organogenesis, was annotated on scaffold 1607 (Grigg *et al.* 2005; Yang *et al.* 2006). Embryonic lethality and defective post-embryonic organ formation have been reported in *Arabidopsis se* mutants, indicating a possible role for *SE* during plant regeneration after successful ME (Prigge and Wagner 2001; Grigg *et al.* 2005; Lobbes *et al.* 2006).

Most intriguing, however, was the annotation of orthologs to two genes encoding distinct domains of the Polycomb Repressive Complex 2 (PRC2), a highly conserved and important regulator of developmental processes, on scaffolds 4385 and 7045 (Förderer *et al.* 2016). The first, *CURLY LEAF* (*CLF*), encodes one of three SET domain proteins, the others being *MEDEA* (*MEA*) and *SWINGER* (*SWN*), which mediate large-scale chromatin remodelling during embryogenic development (Liu *et al.* 2016). In fact, the mannitol stress treatment used prior to barley IMC was found to induce the upregulation of *CLF* in anther tissue (Muñoz-Amatriaín *et al.* 2009a). The second homolog is a *FIE* domain which is associated with *MEA* in the gametophytic- and endosperm-specific configuration of the PRC2. In *Arabidopsis*, *fie* as well as *clf swn* double mutants are unable to terminate the embryogenic phase of germination and proliferate into so-called PcG callus (Chanvivattana *et al.* 2004; Bouyer *et al.* 2011). Furthermore, the PRC2 complex is involved in the negative regulation of the LEC family as well as *WUS* genes, both of which play key roles in somatic and ME (Berger *et al.* 2011; Lowe *et al.* 2016). In fact, *LEC1*, *LEC2* and *FUS3* are overexpressed in *clf swn* double mutants of *Arabidopsis* (Makarevich *et al.* 2006). Indeed, *LEC1* (over-)expression was shown to negatively affect ME in both rapeseed and rye (Gruszczyńska and Rakoczy-Trojanowska 2011; Elahi *et al.* 2016). Interestingly, a homolog of the MADS box gene *AGL26*, was annotated along with *FIE* on scaffold 4385. Several MADS box transcription factors, which are key regulators of developmental processes, are negatively regulated by PRC2 as well (Masiero *et al.* 2011). Ultimately, the distinct phases of in vitro androgenesis are likely to require different levels of PRC2 mediated repression of specific genes (Förderer *et al.* 2016). Quantification or manipulation of the expression of *CLF*, *FIE*, *AGL26* or any of the other candidate genes during different stages of perennial ryegrass in vitro AC could confirm their contribution to successful androgenesis and should determine if and when their expression is most beneficial.

5.4.5 Concluding remarks

Here, we have demonstrated the effectivity of a multi-parental genome-wide association mapping approach in perennial ryegrass and report the first genetic loci associated with the response to in vitro AC. Elucidation of the exact locations of the QTL detected here will, however, require the availability of a more complete perennial ryegrass genome assembly. It can then be ascertained whether the co-localization of several QTL associated with different traits or detected in different years was, in fact, accurately determined using the GenomeZipper (Pfeifer *et al.* 2013). Future studies on the genetic control of androgenic capacity may then focus on these important regions. Of particular interest is a major QTL for green plant regeneration on LG 5 which, if proven to be effective in different genomic backgrounds, is an excellent candidate for further fine mapping approaches. A second major QTL for embryo production on LG 1 was detected in the smaller of the two datasets that were used here, but nevertheless merits additional investigation. Two of the identified candidate genes, *CLF* and *FIE*, are of great potential interest, given their extensively documented involvement in embryogenesis and

organogenesis, although expression studies will have to provide further evidence of their involvement in perennial ryegrass ME (Förderer *et al.* 2016). Presently, our results allow for the development of molecular markers which will enable efficient introgression of androgenic capacity into recalcitrant perennial ryegrass germplasm. The availability of an efficient system for homozygous line production will aid in the establishment of a hybrid breeding system, which should increase the rate of genetic gain in this forage crop of global importance.

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6. Synthesis

6.1 Doubled haploid induction in perennial ryegrass (*Lolium perenne* L.)

In this thesis, evidence for the potential of an in vitro anther culture (AC) method for the efficient production of doubled haploid (DH) perennial ryegrass genotypes has been presented. Using amenable genotypes, hundreds of green plants can be obtained per petri dish of cultured anthers. However, the previously reported genotype-specific and often low androgenic capacity of perennial ryegrass was also confirmed and more work needs to be done before DH induction can be applied economically and on a large scale. Clues as to the genetic control of androgenic capacity, genetic loci influencing the component traits of this ability as well as interesting candidate genes for further study have been reported here and will aid future investigations into the improvement of androgenic ability in perennial ryegrass. Furthermore, the results obtained here could be of significant benefit to the research and breeding communities of related Poaceae species as well.

6.1.1 The genetic control of the component traits of androgenesis

Support for the polygenic and distinct genetic control of the component traits of androgenesis, reported earlier, has been presented in **chapter 4** (Opsahl-Ferstad *et al.* 1994b; Madsen *et al.* 1995). It could be established that additive and dominance effects play a role in the highly correlated embryo and total plant production abilities, while green plant regeneration may be controlled through dominance or by the complementation of recessive alleles between parents. Embryo production and plant regeneration as well as green and albino plant recovery are only slightly correlated and should thus be influenced by distinct genetic factors. Finally, the environment is likely to be of a larger influence on albinism than genetics. The results of the genome-wide association study (GWAS), detailed in **chapter 5**, were in agreement with these findings and provided further means of distinguishing the genetic control of the androgenic traits.

The identification of a relatively large number of QTL with modest effects confirmed the polygenic control of both embryo and albino plant production. Even though both albinism and microspore embryogenesis (ME) are complex phenomena, their manipulation by genetic means seems to be possible (Krzewska *et al.* 2015; Hand *et al.* 2016). A major QTL was detected for green plant regeneration on linkage group (LG) 5, chromosomal homologs of which have yielded major QTL for green plant recovery in four related Poaceae species as well (He *et al.* 1998; Torp *et al.* 2001; Chen *et al.* 2007; Muñoz-Amatriaín *et al.* 2008; Krzewska *et al.* 2012). A second putative major QTL, associated with a significant improvement in embryo production, was detected in the smaller of the two GWAS datasets. Consequently, a higher degree of uncertainty is related to its effect compared to the QTL on LG 5. Nevertheless, apart from traits that are governed by many loci of minor effect, it seems to be possible to affect considerable changes to least one androgenic characteristic by targeting a single genetic factor.

Interestingly, the three QTL detected for total plant production were also associated with embryo production, affirming the high correlation between these aspects as well as suggesting that it may not be necessary to record the former trait (Beaumont *et al.* 1995). Furthermore, the absence of QTL for total plant regeneration indicated that green and albino plant regeneration are genetically distinctly controlled processes. In fact, the QTL for green or albino plant regeneration were uniquely associated with those traits. The total number of regenerated plants, which is a summation of the number of regenerated green and albino plants, may therefore not be a meaningful characteristic to study either.

6.1.2 Marker-assisted introgression of androgenic capacity

One of the most exciting possibilities for the application of the GWAS results is the development of molecular SNP markers to enable rapid introgression of high embryo production and green plant

regeneration ability into recalcitrant genotypes. However, the actual effectivity of such a programme needs to be assessed first (Madsen *et al.* 1995). Highly androgenic genotypes identified here may be crossed with elite breeding germplasm so that the response to AC of the progeny can be ascertained and the predictive value of any markers evaluated. For this purpose, it is worth investigating whether further improvements of androgenic ability are possible via pair- or polycrosses between highly responsive plants of this study, again using markers to ascertain which beneficial alleles are being combined.

6.1.3 Validation of molecular markers derived from the QTL

Apart from introgression approaches to improve androgenic capacity, molecular markers may be used to screen perennial ryegrass breeding germplasm to identify responsive individuals. A marker-based assay is likely to be cheaper than assessing androgenic ability empirically in the laboratory, which has been estimated to take one hour of lab work per genotype (Tuvesson *et al.* 2000). In addition, pre-screening germplasm before it is entered into costly DH production pipelines avoids wasting time and money on recalcitrant material. The putative major QTL for green plant regeneration on LG 5 is of particular interest in this context. A crucial first step before such screening approaches are attempted, however, is the validation of any markers in germplasm unrelated to the plant material that was used to create them.

Three reports of androgenicity-related QTL validation have been published, namely in rice, barley and wheat (**Suppl. Table S1.1**). A marker on rice chromosome 10 that was detected in 164 recombinant inbred lines (RILs), could be used to distinguish bad (< 3%) and good (>10%) producers of embryo-like structures (ELS) with green plant regeneration capacity per cultured anther in 43 cultivars (Kwon *et al.* 2002). In the barley study, QTL associated with green plant production were detected on chromosomes 3H and 5H in a bi-parental mapping population, but only the QTL on 3H could be confirmed in 20 elite cultivars (Muñoz-Amatriaín *et al.* 2008). Nielsen *et al.* (2015) reported that several QTL, identified in a bi-parental population, were rare in 94 European wheat varieties and cultivar 'Svilena', the highly responsive parent, was the only one carrying all four beneficial alleles (Nielsen *et al.* 2015). Whether the situation in perennial ryegrass reflects any of these results remains to be determined.

The GWAS in this thesis was performed on preselected populations exhibiting extreme phenotypes and therefore probably containing high frequencies of uncommon alleles. Since androgenic ability, particularly green plant production, is an exceedingly rare trait in perennial ryegrass germplasm, it follows that the beneficial alleles identified here are simply not present at high frequencies (Andersen 2003). Even if they are, their effects may be masked by other genetic factors influencing androgenic capacity, which would inhibit QTL validation. Most importantly, however, the genetic distance between the markers that will be developed from the QTL detected here and the causative genes is unknown. The greater that distance is, the smaller the chance that linkage between the marker and the relevant gene(s) exists in the highly heterozygous natural populations, ecotypes, breeding material or varieties of perennial ryegrass, which are characterized by short linkage disequilibrium (LD) distances (Roldán-Ruiz and Kölliker 2010; Ruttink *et al.* 2013). It has therefore been proposed to follow candidate gene-based marker-assisted approaches instead (Skøt *et al.* 2007; Smith *et al.* 2009). Whether any of the polymorphisms associated with androgenic traits in this study lie within causal genes remains to be determined. An investigation of the responses to AC of DH populations derived from single, highly androgenic genotypes identified here, may elucidate this matter.

6.1.4 Candidate genes

Several intriguing genes have been annotated in proximity to the QTL detected in the GWAS, such as *ISOPRENYLCYSTEINE METHEYLESTERASE-LIKE* 2 (*ICME-LIKE2*), *VIP HOMOLOG 1* (*VIH1*), *SERRATE* (*SE*), MADS-box gene *AGL26* and Polycomb Repressive Complex 2 (PRC2) domain protein encoding genes

CURLY LEAF (*CLF*) and *FERTILIZATION INDEPENDENT ENDOSPERM* (*FIE*). Of particular interest are the genes that are known regulators of developmental processes, *SE*, *AGL26*, *CLF* and *FIE*, and it could be worthwhile to study and manipulate their expression patterns during in vitro AC in perennial ryegrass (Radoeva and Weijers 2014). It has been shown that ectopic or over-expression of developmental regulators like *LEAFY COTYLEDON 1* (*LEC1*), *LEC2*, *SOMATIC EMBRYOGENESIS RECEPTOR KINASE 1* (*SERK1*), *WUSCHEL* (*WUS*) and *BABY BOOM* (*BBM*) can trigger embryogenesis (Lotan *et al.* 1998; Hecht *et al.* 2001; Stone *et al.* 2001; Boutilier 2002; Zuo *et al.* 2002). In fact, a recent major breakthrough in monocot post-transformation plant regeneration from tissue culture was achieved through the co-expressing morphogenic growth regulators *WUS2* and *BBM* (Lowe 2016). Embryogenesis and plant regeneration were significantly increased in recalcitrant genotypes and explants so that unprecedentedly high plant regeneration frequencies were obtained in maize (*Zea mays* L.), sorghum (*Sorghum bicolor* L.), sugarcane (*Saccharum officinarum* L.) and *indica* rice (*Oryza sativa* L.). Conversely, mutation or reduced expression of factors repressing embryogenesis, such as *PICKLE* (*PKL*) and multiprotein regulatory complexes like PRC2, can stimulate embryo development as well (Ogas *et al.* 1999; Bouyer *et al.* 2011; Förderer *et al.* 2016). Both *PKL* and PRC2 are involved in the negative regulation of some of the above mentioned stimulators of embryogenesis, including the *LEC* gene family. It thus follows that if the optimal timing and expression levels of the genetic regulators of the distinct developmental processes of androgenesis can be elucidated in perennial ryegrass, major increases in the efficiency of ME and plant regeneration may be achieved.

6.2 Characteristics of the anther culture derived doubled haploids

Ultimately, the practical utility of perennial ryegrass DH production is determined by several crucial characteristics of the AC-derived green regenerants themselves. Although the average spontaneous diploidization frequency of 50-80% as well as the negligible recovery of 0.2-0.3% heterozygotes has already been established for perennial ryegrass, three important aspects require further investigation and will be discussed in the next sections (Andersen *et al.* 1997). The approximate number of anthers that need to be cultured to obtain sufficient germplasm for each specific application of the DHs may then be calculated. In addition, the minimum AC yield or efficiency, allowing for practically feasible large-scale DH induction programmes, can thus be established and used as a breeding target for the introgression of androgenic capacity into recalcitrant germplasm.

6.2.1 Clones

The first consideration is the incidence of clones. In the absence of the induction of direct ME, androgenic structures, such as the ELS recovered during perennial ryegrass AC, are produced that frequently regenerate multiple green shoots. In a recent study on IMC in triticale, it was reported that 80% of such shoots are in fact clones (Oleszczuk *et al.* 2014). During the AC performed in the current study, it was notably difficult to establish the precise dimensions of the ELS. Clumps of structures of distinct microspore origin may have been classified as a single ELS, or conversely, highly callogenic ELS may have been divided into several genetically identical parts (Seldimirova and Kruglova 2015). Molecular markers should be used to determine the incidence of clones among AC derived perennial ryegrass DHs. If the incidence is high, strategies may need to be developed to ensure single microspore origin of the recovered DH, such as the retention of only one green plant from each ELS. This could dramatically lower the attainable green plant yield.

6.2.2 Tissue culture-related somaclonal variation and segregation bias

Secondly, two phenomena inherent to the in vitro DH induction process can have a significant influence on the genomic makeup of the recovered plants. Firstly, tissue culture-related somaclonal variation can generate genetic or epigenetic mutations, causing changes to the phenotypes of the regenerated plants (Bairu *et al.* 2011). Indeed, studies comparing somatic and AC embryogenesis found

comparable results for both explant types and an average of 6% and 10% nucleotide and epigenetic somaclonal variation between regenerants in barley and triticale, respectively (Bednarek *et al.* 2007; Machczyńska *et al.* 2014). In perennial ryegrass AC, the presence of a callogenic, ELS culture phase instead of direct ME could confer increased vulnerability to somaclonal variation (Seguí-Simarro 2016). It depends on the application of the DHs whether somaclonal variation is a desirable side effect which can introduce potentially beneficial variation into breeding programmes, for instance, or a hindrance when robust mapping populations are required, for example (Carloni *et al.* 2017).

The second phenomenon concerns the significant marker segregation bias that is often reported in DHs derived from in vitro cultures, causing an overrepresentation of the alleles of the parental genotype with the highest androgenic capacity (Devaux and Zivy 1994; Sayed *et al.* 2002; Cistué *et al.* 2005; Ferrie and Möllers 2010). In fact, a comparison of wide-hybridization and AC to produce barley DHs revealed a greater segregation distortion with the latter method (Cistué *et al.* 2011). This effect may be due to marker linkage to genes that are either detrimental or beneficial to survival of the in vitro culture process. Selection against the former type of allele could help to eliminate inbreeding depression, selection of the latter, while improving androgenic capacity, has an unknown effect on agronomic performance (Wang *et al.* 2005; Strigens *et al.* 2013). The proportion of distorted markers can be as high as 44%, although they tend to cluster in segregation distortion regions (Li *et al.* 2010; Bélanger *et al.* 2016). It was only recently determined, in barley, that the selective forces that cause these biases act during embryo formation and plant regeneration. No significant segregation bias was detected in the population of microspores, either prior or after the stress induction treatment (Bélanger *et al.* 2016). Segregation distortion in perennial ryegrass DHs obtained by AC has been reported, although its full extent is still unknown (Hayward *et al.* 1990; Andersen *et al.* 1997). Since this phenomenon causes uncertainty in the estimated gains from selection, may reduce the chance of recovering beneficial allelic combinations and can distort genetic and QTL mapping approaches, it has to be taken into consideration.

6.2.3 Fertility and growth vigour

Andersen *et al.* (1997) defined two types of inbreeding depression, affecting vegetative growth and fertility in perennial ryegrass DHs, respectively. The practical use of DHs, particularly in breeding programmes, depends on their growth vigour, male and female fertility and seed production ability. All of these aspects commonly decline with increased levels of homozygosity due to inbreeding depression, especially in obligate outbreeders (Bean and Yok-Hwa 1972; Thorogood *et al.* 2005; Charlesworth and Willis 2009). Low growth vigour of perennial ryegrass DHs has been reported previously and confirmed, although not quantified, here. The high levels of inbreeding depression displayed by perennial ryegrass have, in fact, led some to question the utility of inbred line production for hybrid breeding (Brummer 1999). Indeed, in a field study on 140 DHs, an average forage yield of only 20-21% and an average seed yield of just 8-12% of the yield of their three parental clones was reported (Opsahl-Ferstad 1993). Furthermore, the fact that vigorous growth does not guarantee good fertility was demonstrated in a topcross experiment of 75 vigorously growing DH and five cultivar pollen donors. Seed yield of the DHs averaged at 0.62 g and only 20% of the plants produced enough seed for subsequent field evaluation of the topcross offspring (Andersen *et al.* 1997). In a study on rye DHs, which also suffer from severe inbreeding depression, it was calculated that only 10-36% of AC derived plants were suitable for use in research or breeding (Tenhola-Roininen *et al.* 2006).

While vigorous and fertile perennial ryegrass DHs have been recovered, additional investigation of their characteristics and how to improve them is needed. Selection against inbreeding depression or among DHs or inbred lines can enhance their performance. Indeed, during the many decades of maize hybrid breeding, inbred yields have increased significantly more than hybrid yields (Hallauer *et al.*

2010). Similarly, the performances of inbred rye lines have steadily improved since hybrid rye breeding started in the 1970s (Schlegel 2016). Interestingly, there are some indications that higher ploidy level AC-derived plants exhibit less severe inbreeding depression (Andersen 2003). An investigation of the homozygous tetraploids recovered from AC of diploid perennial ryegrass genotypes, or indeed artificially chromosome doubled DHs, may be worthwhile. Ultimately, significantly higher recovery rates of vigorous and fertile perennial ryegrass DHs need to be realised. An effective method to screen the green regenerants from in vitro DH induction for growth vigour and fertility at a very young stage, perhaps through some novel incarnation of genomic selection, would eliminate the need for expensive field trials (Daetwyler *et al.* 2015; Byrne *et al.* 2017).

6.3 Isolated microspore culture of perennial ryegrass

Although an effective AC protocol is available for perennial ryegrass, the development of an IMC platform will confer significant advantages (Ferrie and Caswell 2011). An improved regeneration frequency when IMC instead of AC was used resulted in a 3.7 and 9.3 fold higher green plant yield in wheat and barley, respectively (Holme *et al.* 1999; Li and Devaux 2005). Embryo production was 9.2 fold higher in triticale IMC, although a markedly lower incidence of albinism made AC the more productive method overall (Lantos *et al.* 2014). In a commercial context, however, where time and cost effectivity may outweigh method efficiency, the outstanding scalability and flexibility of IMC may make it the method of choice (Castillo *et al.* 2014).

Microspores are usually isolated by mechanically crushing entire spikes followed by a series of filtration steps, which is considerably less laborious than manual anther recovery from individual flowers. Distinct developmental stages can be selected via density gradient centrifugation of the isolated microspores to ensure optimal and uniform induction of ME. At this phase, microspore viability tests, such as non-destructive impedance flow cytometry, may be carried out so that adjustments to the pre-treatment stresses can be made accordingly (Heidmann *et al.* 2016). In addition, the direct feedback provided by the naked microspores accelerates the determination of optimal culture procedures to ensure successful ME and a large number of media and methods may be accurately compared in small volumes of the liquid cultures (Holme *et al.* 1999). Selection of competent embryos or ELS prior to culture on the regeneration medium, which has been shown to improve plant production efficiencies in rice and wheat, is facilitated by the liquid IMC induction medium (Park *et al.* 2013; Rubtsova *et al.* 2013; Giri and Praveena 2014).

A single scientific text mentions plant regeneration using IMC in perennial ryegrass but, except for an optimum 2,4-D concentration of 0.38-0.75 mg L^{-1}, a full protocol has never been published (Andersen *et al.* 1997). Similar to the recent successes obtained in oat, an adaptation of modern cereal IMC protocols should enable the development of an efficient IMC method for perennial ryegrass (Ferrie *et al.* 2014). The availability of such a protocol would not only increase DH production speed and flexibility, but also allow for the adaptation of exciting microspore transformation, genome editing, live imaging and gene expression study procedures from crops such as wheat, barley, rapeseed and tobacco to perennial ryegrass (Daghma *et al.* 2014; Eudes *et al.* 2014; Gurushidze *et al.* 2014; Seifert *et al.* 2016).

6.4 Key requirements for perennial ryegrass hybrid breeding

6.4.1 Multiplication of doubled haploid (DH) lines

Since an effective self-incompatibility (SI) system as well as a marked sensitivity to inbreeding depression prevent perennial ryegrass inbred line production through repeated cycles of selfing (Cornish *et al.* 1979; Brummer 1999), DH production is a promising alternative. The method is fast and has reduced inbred line production in species like rice and maize by 5-7 years (Röber *et al.* 2005; Mishra

and Rao 2016). Remarkably, the fact that DH production does not allow selection at every cycle of selfing, was the reason for the CIMMYT wheat breeding programme to continue using classical shuttle breeding in spite of significant progress in DH induction protocols (Li *et al.* 2013). Such considerations play no role in perennial ryegrass due to a rapidly increasing degree of inbreeding depression in each successive iteration of self-fertilization. However, contrary to autogamous species like maize, wheat, barley or rice, the multiplication of DHs to obtain sufficient material for test crosses and seed production is challenging. Pseudo-compatibility can be induced via heat or chemical treatments, but the introgression of genetic self-fertility (SF) may be more economically feasible for this purpose (reviewed in Do Canto *et al*. 2016). A major QTL for SF, termed the *T* locus, has been mapped on perennial ryegrass LG 5 and should enable the development of self-fertile DHs (Thorogood *et al.* 2005; Aguirre *et al.* 2013).

6.4.2 Heterosis and heterotic groups

It is questionable to what extent heterosis is exploited in modern synthetic perennial ryegrass varieties, which are made up of polycrosses of multiple, distinct, heterozygous elite genotypes. In fact, dry matter yields decrease during seed multiplication cycles of such synthetics, for example when less than seven or five parents are used to create diploid and tetraploid cultivars, respectively (Posselt 2003; Boller *et al.* 2016). Molecular markers may be used to avoid inbreeding depression during synthetics' seed production by ensuring sufficient genetic distance between the parents (Kölliker *et al.* 2005). Making use of more than one genepool in breeding programmes, which is not yet common practice, should not only increases the genetic distance between polycross parents but also allow for some exploitation of heterotic effects in perennial ryegrass breeding (Posselt 2010). Similarly, chance or semi-hybrids may be produced from genetically distinct elite populations that are crossed just once for seed production, thus avoiding the inbreeding associated with synthetic varieties and producing a proportion of heterotic, hybrid seed (Brummer 1999). A broadening of the perennial ryegrass breeding genepool is certainly possible, since genetic variation in ecotype collections of perennial ryegrass is extensive and it is not uncommon to find high yielding populations among them (Wilner *et al.* 2010). Furthermore, methods to group genotypes into heterotic groups have been applied, leading, for instance, to the identification two genepools in individual studies on Irish, German and European germplasm (Bolaric *et al.* 2005; Brazauskas *et al.* 2011; Barth *et al.* 2015). While some publications on perennial ryegrass breeding strategies make no mention of genetic distance, heterotic groups or hybrid breeding at all (Casler and Brummer 2008; Conaghan and Casler 2011), a renewed interest in such matters is apparent in, for example, New Zealand (Barrett *et al.* 2010; Connor *et al.* 2015) and Australia (Pembleton *et al.* 2015; CRC Dairy Futures 2016). All in all, genetically distinct, heterotic pools can be defined and homozygous lines may be produced using DH induction, which makes the absence of an effective pollination control system the main hindrance to the realization of hybrid breeding.

6.4.3 Pollination control and hybrid breeding

Cytoplasmic male sterility (CMS) has been used to control pollination in hybrid seed production programmes of crops such as maize, onion, sunflower, rice, *Brassica* species and rye (Havey 2004; Cheng *et al.* 2007; Geiger and Miedaner 2009). Two CMS systems have been introduced into perennial ryegrass, one via interspecific hybridization with *L. multiflorum* and the second using an intergeneric cross with *Festuca pratensis*, but neither of them has been fully characterized yet (Wit 1974; Connolly and Wright-Turner 1984; Islam *et al.* 2014). While the hybrid plant itself does not need to be male fertile in forage, vegetable or fruit crops because their seeds are not the final marketable product, fertility restoration is required for the maintenance and improvement of the CMS seed parent. *Restoration of fertility* (*Rf*) genes are therefore crucial to hybrid seed production systems based on CMS, however, *Rf* genes have not yet been identified in perennial ryegrass. Fortunately, a recently developed in silico pipeline for the identification of such genes will soon yield markers for *Rf* loci

(Timothy Sykes, personal communication; Sykes *et al*. (2016)). Even so, their ultimate usefulness depends on whether perennial ryegrass *Rf* genes are linked to any undesirable phenotypes, as was the case with increased ergot infection susceptibility of certain rye *Rf*, as well as on how common they are in breeding germplasm (Hackauf *et al.* 2017). It has been suggested that the introgression of CMS and *Rf* genes into elite germplasm may be prohibitively laborious and expensive (Pembleton *et al.* 2015). Nevertheless, DH induction combined with molecular markers can be used to select for the required allelic combinations as well as against the undesired genetic background and accelerate introgression programmes of this kind (Tuvesson *et al.* 2006; Guo *et al.* 2009b; Lübberstedt and Frei 2012).

Given that perennial ryegrass inbred lines suffer from inbreeding depression, the production of top-cross hybrids may be the most feasible strategy. The CMS system used in rye, for instance, following a $(A_{CMS} \cdot B) \cdot Syn_{Rf}$ scheme, could be a good start. Importantly, the rye CMS seed parent is, in fact, a single-cross hybrid between two genetically distant inbred lines to guarantee sufficient vigour and seed production (Schlegel 2016). As stated above, fertility restoration in the pollen parent is not required for forage crops, although a selection against *Rf* genes in the germplasm used to maintain and improve the CMS lines is important to guarantee pollen sterility and thus ensure the purity of the hybrid seed (Islam *et al.* 2014). Whether the pollen parent in a perennial ryegrass hybrid breeding scheme is a synthetic population, DH or indeed a single-cross hybrid as well, ultimately depends on the performance of the homozygous germplasm. The latter situation would actually be classified as double-cross hybrid production, which is known to produce more heterotic tetraploids hybrids than the single-cross method (Riddle and Birchler 2008).

An alternative pollination control strategy for the production of perennial ryegrass hybrids exploits its two-locus, *S* and *Z*, self-incompatibility (SI) system (England 1974; Posselt 1993; Pembleton *et al.* 2015). These types of strategies require exact determination of *S* and *Z* alleles, for instance via fast and cheap high resolution melting (HRM) curve analysis (Arias Aguirre *et al.* 2014; Manzanares *et al.* 2016a). The main premise is that two heterotic pools, which differ in their allelic constitution at the S and Z loci, are crossed so that their progeny consists of a high percentage, up to 83%, of F1 hybrids (Pembleton *et al.* 2015). To obtain 100% F1 hybrid progeny, however, the SI system would need to be temporarily repressed in order to allow sufficient multiplication of the parental populations, DHs or inbred lines, prior to hybrid seed production. For this purpose, the phenomenon of pseudo-compatibility could be exploited, which can confer selfing rates as high as 31% at high temperatures. However, the heated greenhouse environment required during such selfings may be prohibitively expensive for large-scale use (Wilkins and Thorogood 1992). A hybrid breeding system based on CMS and *Rf* genes may therefore be a realistic system for the production of exclusively F1 hybrid seed.

Nevertheless, the SI system enables the exploitation of heterosis without the need for laborious CMS or *Rf* gene introgression and may be combined with DH production to capture unprecedented heterotic effects in perennial ryegrass varieties. If DHs are induced from elite germplasm and typed for *S* and *Z* alleles using molecular tools, homozygous material with distinct, fixed SI determinants becomes available. In this scenario, the breeder has complete control over any subsequent crosses. Heterotic groups of DHs carrying distinct *S* and *Z* alleles could be created, for instance, so that complete hybridization between populations is guaranteed. Contrary to the breeding strategy proposed by Pembleton *et al.* (2015), where cross compatibility within the heterotic pools capped F1 hybrid production at 83%, all harvested seed will be derived from an inter-population cross. Furthermore, the inbreeding depression of the DHs should be alleviated from the very first cross, so that sufficient seed may be produced. If flowering time synchronicity permits, efficient DH induction and marker-based characterization of the *S* and *Z* alleles, allow perennial ryegrass breeders to generate highly designed crosses, populations and varieties that effectively exploit any available heterosis.

6.5 Concluding remarks

This thesis documents the initiation of a modern reboot of the development of an efficient DH production system for an economically significant forage species, perennial ryegrass. Examples of the diverse and exciting applications of DH technologies have been discussed, which illustrate their unprecedentedly high potential to advance research and breeding at this time. Before wide-spread and large-scale DH induction becomes practically and economically feasible, however, further efforts are required to 1) elucidate the value of any molecular markers for androgenic ability introgression or germplasm screening approaches, 2) evaluate the incidence of clones, allelic distortion, somaclonal variation and inbreeding depression of the AC-derived DHs, and, perhaps, 3) switch from an AC to an IMC platform for increased efficiency. Fortunately, the plant material, experimental methodology and scientific knowledge to overcome these outstanding challenges are now available so that efficient DH production may soon be added to the arsenal of the research and breeding tools of the forage and turf grass community.

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8. Supplementary information

8.1 Supplementary information for chapter 1

Supplementary Table S1.1 Overview of marker-trait, or QTL, studies on androgenic response of cereal crop species. The culture procedure, plant material characteristics and androgenic response (between brackets) as well as the used markers are summarised. If not stated otherwise, the experiments were carried out at one time point. In some cases, multiple simultaneous experiments or repetitions of the same experiments in different environments or points in time were performed and QTL detected in them are designated letters. Terms such as embryos, embryogenic calli, calli, androgenic structures and the like are referred to as embryo-like structures here. AC – anthers cultured; AFLP – amplified fragment length polymorphism; AP – albino plants; DArT – diversity arrays technology; DH – doubled haploid; ELS – embryo-like structures; GP – green plants; LG – linkage group; PPRA – plants per responsive anther; tELS – ELS transferred to regeneration medium; RA – responsive anthers; RAPD – random amplification of polymorphic DNA; RFLP – restriction fragment length polymorphism; SSR – simple sequence repeat; STS – sequence-tagged site.

Authors(s)	Experiment summary	Chromosome (locus) or marker	Trait[cross/experiment]	Variation explained (%)
Manninen (2000)	Barley (*Hordeum vulgare* L.) anther culture	2H (1)	PPRA	
		2H (2)	PPRA	
	31 DHs of F2 of Rolfi x Botnia (both spring, six-rowed)		RA (%)	
	94 RAPD and 14 RFLP markers	2H (3)	PPRA	
		2H (4)	RA (%)	
		3H (1)	PPRA	
		3H (2)	PPRA	
		3H (3)	PPRA	
		4H (1)	RA (%)	
		4H (2)	RA (%)	
		4H (3)	RA (%)	
			Diploid GP (%)	
Chen *et al.* (2007)	Barley anther culture	2H	ELS/100AC	41
			AP/100AC	25
	41 DHs of Dobla (winter/spring, six-rowed, moderate) x Igri (winter, two-rowed, good)	5H	GP/100AC	18
			GP (%)	21

Authors(s)	Experiment summary	Chromosome (locus) or marker	Trait[cross/experiment]	Variation explained (%)
	35 RAPD, 42 SSR and 13 STS markers	6H	ELS/100AC	12[1]
			GP/100AC	33[2]
Muñoz-Amatriaín *et al.* (2008)	Barley anther culture	3H	GP/100AC	26[3]
			AP/100AC	17
	100 DHs of Igri x DH46 (DH of Igri x Dobla, low)		GP (%)	28
	47 AFLP, RAPD, STS and SSR markers	5H (1)	GP/100AC	21[4]
			GP (%)	37[5]
		5H (2)	AP/100AC	32[6]
		6H	ELS/100AC	25
Torp *et al.* (2001)	Wheat (*Triticum aestivum* L.) anther culture	2AL	GP (%)[A]	
		2BL (1)	GP (%)[A]	
	A) 50 DHs of Ciano (spring, good) x Walter (spring, low)	2BL (2)	GP (%)[A]	
	B) 49 DHs of Ciano x Benoist (winter, good)	5BL	GP (%)[A, B]	[8,9]
	256 AFLP, microsatellite and RFLP markers			
	3-4 repetitions, every 2 months[7]			
Nielsen *et al.* (2015)	Wheat isolated microspore culture (50 000 mL^{-1})	1B	GP/spike	34
			AP/spike	31
	40 F3 of Svilena (winter, good) x Jensen (winter, low)	5A	Embryos/spike	20
	2500 marker DArT array	5B	Embryos/spike	10[10]
		7B	GP/spike	24[11]
			AP/spike	21[12]
Kiviharju *et al.* (2004)	Oat (*Avena sativa* L.) anther culture	Marker OPX-11	GP/100AC	
			AP/100AC	

Authors(s)	Experiment summary	Chromosome (locus) or marker	Trait[cross/experiment]	Variation explained (%)
		Marker OPC-10	GP/100AC	
	38 F2 of Puhti (low) x red oat (*A. sterilis*, acc. CAV 2648, good)		GP/tELS	
			Plants/tELS	
	186 RAPD markers		Plants/100AC	
		Marker OPC-14	GP/100AC	
			Plants/100AC	
			AP/100AC	
			tELS/100AC	
		Marker OPY-03	tELS/100AC	
González *et al.* (2005)	Triticale (*× Triticosecale*) anther culture	1B	GP/1000AC	17
		1R	GP/1000AC	17
	73 DHs from Torote (low) x Presto (good)	3R	GP (%)	22
		4R	ELS/100AC	12
	18 RAPD, 40 microsatellite, 276 AFLP and 22 SSR markers		GP/1000AC	14
		6B	ELS/100AC	10[13]
			Plants/100ELS	6
		7R	GP/1000AC	14[14]
		LG1	Plants/100ELS	13[15]
Krzewska *et al.* (2012)	Triticale anther culture	4A (1)	Plants/tELS[A]	16
		4A (2)	GP/tELS[A, C]	13
	90 DHs from Saka 3006 (inbred line, low) x Modus (good)	4R (1)	ELS/100AC[C]	12-16
			GP/100AC[C]	22
	155 SSR, 29 AFLP and 1385 DArT markers		Plants/100AC[A, B]	8-18
	A) April 2009	4R (2)	ELS/100AC[C]	16
			GP/100AC[C]	20
	B) January		Plants/100AC[B]	7-17
		4R (3)	GP/100AC[C]	21

Authors(s)	Experiment summary	Chromosome (locus) or marker	Trait[cross/experiment]	Variation explained (%)
			Plants/100AC[B]	20
	C) October 2010	5A (1)	ELS/100AC[A, C]	13-14
			GP/100AC[B]	12
		5A (2)	ELS/100AC[B]	11
		5R (1)	ELS/AC[A, B]	9-16
			Plants/100AC[A]	8
		5R (2)	ELS/100AC[A, B]	7-12
			Plants/100AC[B]	13
		5R (3)	GP/100AC[A]	13
		7R	ELS/100AC[A,B]	5-13
			Plants/100AC[B]	13
Krzewska *et al.* (2015)	Triticale anther culture	2AL.2BL	AP/100ELS[B]	
		2BS.6AL	AP/100ELS[B]	
	90 DH from Saka 3006 (inbred line, low) x Modus (good)	2RS.3R	AP/100AC[B]	
		3B (1)	AP/100ELS[A]	8
	155 SSR, 28 AFLP and 1385 DArT markers	3B (2)	AP/100ELS[B]	
	A) Composite interval mapping (CIM)	4B	AP/100AC[A]	18
		4R (1)	AP/100AC[A]	12
	B) Single marker model	4R (2)	AP/100AC[B]	
		4R (3)	AP/100AC[B]	
		4R (4)	AP/100AC[B]	
		5R (1)	AP/100ELS[A]	17
		5R (2)	AP/100ELS[A]	11
		5R (3)	AP/100ELS[A]	13
		7R	AP/100AC[A]	17
Yamagishi *et al.* (1998)		1	RA (%)	
	Rice (*Oryza sativa* L.) anther culture	10	ELS with ≥ 1 GP (%)	

Authors(s)	Experiment summary	Chromosome (locus) or marker	Trait[cross/experiment]	Variation explained (%)
	19 DHs from Nipponbare (*japonica*, good) x Milyang 23 (*Tongil*, moderate) 55 RFLP markers			
He *et al.* (1998)	Rice anther culture 110 DHs from ZYQ8 (*indica*, low) x JX17 (*japonica*, good) 187 RFLP markers	1 (1)	ELS with ≥ 1 GP (%)	14
		1 (2)	ELS with ≥ 1 GP/AC	10
		6	RA (%)	10
		7	RA (%)	12
		8	RA (%)	12
		9 (1)	ELS with ≥ 1 GP (%)	21
			ELS with ≥ 1 GP/AC	17
		9 (2)	ELS with ≥ 1 AP (%)	28
		10	ELS with ≥ 1 GP/AC	15
			RA (%)	10
		12	RA (%)	21
Kwon *et al.* (2002)	Rice anther culture 164 RILs from Milyang 23 (*Tongil*, moderate) x Gihobyeo (*japonica*, good) 212 RFLP, 231 AFLP, 86 SSR, 5 isozyme and 2 morphological markers 3 distinct cold pre-treatment methods (A, B, C)	3	ELS with ≥ 1 GP/AC[A]	10
			ELS with ≥ 1 GP/AC[B]	11
		10[18]	ELS with ≥ 1 GP/AC[A]	9[16]
			ELS with ≥ 1 GP/AC[B]	8[17]
			ELS with ≥ 1 GP/AC[C]	18
Cowen *et al.* (1992)	Maize (*Zea mays* L.) anther culture	1	ELS/100AC	

Authors(s)	Experiment summary	Chromosome (locus) or marker	Trait[cross/experiment]	Variation explained (%)
	98 Syn1 from B73 (inbred line, low) x 139/39-05 (S_4 line, good)	3	ELS/100AC	4
		9	ELS/100AC	4[19]
	75 RFLP markers	10	ELS/100AC	[20, 21]
Wan *et al.* (1992)	Maize anther culture	1 (1)	ELS/100AC	
	Inbreds H99 (low), FR16 (moderate), Pa91 (moderate), H99 x FR16, H99 x Pa91 and FR16 x Pa19	1 (2)	ELS/100AC	
		2 (1)	ELS/100AC	
		2 (2)	ELS/100AC	
	35-52 RFLP markers	3	ELS/100AC	
		6	ELS/100AC	
	Data from 2-4 years was pooled[22]	8	ELS/100AC	
Murigneux *et al.* (1994)	Maize anther culture	1	ELS/100AC[B]	12
	A) 48 DHs from DH5 (good) x DH7 (good)	3	RA (%)[A]	19
	B) 96 DHs from A188 (low) x DH7	4 (1)	RA (%)[B]	16
	C) 95 DHs from R6 (low) x DH99 (good)		ELS/100AC[B]	19
	109 RFLP markers		Plants/100AC[B]	19
		4 (2)	ELS/100AC[A]	23
			Plants/100AC[A]	10
		5 (1)	RA (%)[B]	12
			ELS/100AC[B]	14
		5 (2)	RA (%)[C]	11
		7	RA (%)[C]	11
		8	RA (%)[C]	18
			ELS/100AC[A]	20
		9	Plants/100ELS[B]	12
		10	RA (%)[C]	10

Authors(s)	Experiment summary	Chromosome (locus) or marker	Trait[cross/experiment]	Variation explained (%)
Beaumont *et al.* (1995)	Anther culture	1 (1)	ELS/100AC[B, C]	
		1 (2)	ELS/100AC[B]	
	A) 45 F2 from H99 (low) x Pa91 (moderate)	1 (3)	ELS/100AC[C]	
		3	ELS/100AC[A]	
	B) 24 F2 from H99 x FR16 (moderate)	5	ELS/100AC[A, B]	
	C) 31 F2 Pa91 x FR16	7 (1)	ELS/100AC[B]	
		7 (2)	ELS/100AC[C]	
	80 RFLP markers	8	ELS/100AC[A]	

[1] A two QTL model for ELS/100AC explained 52% of the phenotypic variation.
[2] A two QTL model for GP/100AC explained 51% of the phenotypic variation.
[3] This QTL was also significantly associated to GP/100AC in 30 elite barley cultivars, explaining 20% of phenotypic variation.
[4] A two QTL model for GP/100AC explained 47% of the phenotypic variation.
[5] A two QTL model for GP (%) explained 65% of the phenotypic variation.
[6] A two QTL model for AP/100AC explained 49% of the phenotypic variation.
[7] The environment accounted for 15% of the variation.
[8] A four QTL model for GP (%) explained 80% of the phenotypic variation in cross A.
[9] This QTL for GP (%) explained 31% of phenotypic variation in cross B.
[10] A two QTL model for embryos/spike explained 41% of the phenotypic variation.
[11] A two QTL model for GP/spike explained 53% of the phenotypic variation.
[12] A two QTL model for AP/spike explained 55% of the phenotypic variation.
[13] A two QTL model for ELS/100AC explained 30% of the phenotypic variation.
[14] A four QTL model for GP/1000AC explained 46% of the phenotypic variation.
[15] A two QTL model for plants/100ELS explained 24% of the phenotypic variation.
[16] A two QTL model for ELS with ≥ 1 GP/AC explained 18% of the phenotypic variation in experiment A.
[17] A two QTL model for ELS with ≥ 1 GP/AC explained 18% of the phenotypic variation in experiment B.
[18] This marker could be used to distinguish good (> 10%) from bad (< 3%) responders in 43 rice cultivars (30 *japonica*, 13 *indica*), 50 F2 from MG RI036 (good) x Milyang 23 and 50 F2 from MG RI036 x IR 36 (low).
[19] A two QTL model including the QTL on chromosomes 3 and 9 for ELS/100AC explained 47% of the phenotypic variation.
[20] A two QTL model including the QTL on chromosomes 1 and 10 for ELS/100AC explained 17% of the phenotypic variation.
[21] A four QTL model for ELS/100AC explained 57% of the phenotypic variation.

[22] The authors do not comment on any potential variation between years.

8.2 Supplementary information for chapter 4

Supplementary Table S4.1 Response of the paircross parents of the populations evaluated in this study to in vitro doubled haploid (DH) induction and their androgenic classification. This data has been generated approximately two decades ago at the research and breeding station of DLF A/S in Store Heddinge, Denmark.

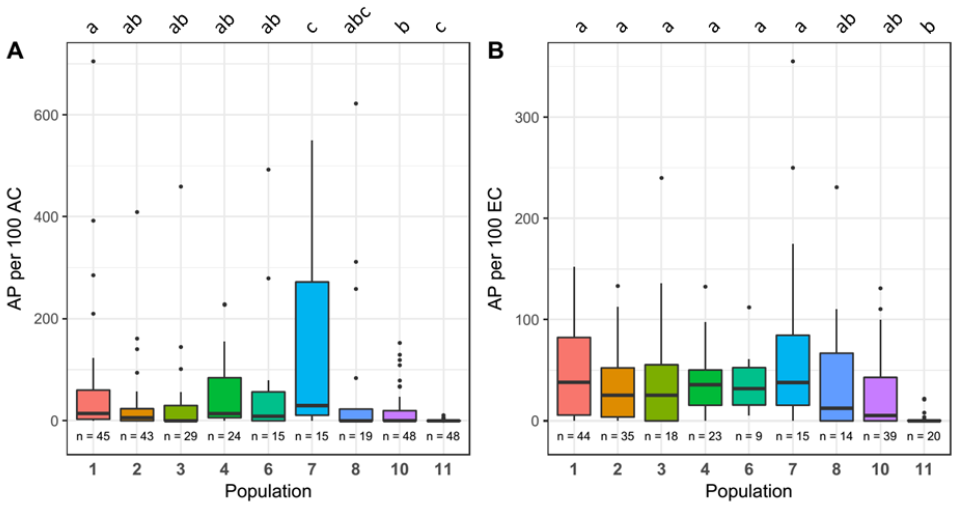
Paircross parent	ELS per 100 AC	Plants per 100 AC	Plants per 100 EC	GP per 100 AC (%)	GP per 100 EC	Androgenic characteristics
Androgenic						
P2	283	168	59	98 (58)	35	Many GP
P102	913	357	39	183 (51)	20	Many GP
P169	906	592	65	506 (86)	56	Many GP
Non-androgenic						
P10	510	171	33	0	0	Many albinos, no GP
P48	527	137	26	0	0	Many albinos, no GP
P133	833	188	23	0	0	Many albinos, no GP
P144	556	103	19	0	0	Many ELS, no plants
P175	0	0		0		No ELS, no plants

AC – anthers cultured; ELS – embryo-like structures; EC – ELS cultured; GP – green plants

Supplementary Table S4.2 Differences between the responses of the genotypes grouped by common non-androgenic paircross parent (top) or common androgenic paircross parent (bottom). The combination with a significantly higher or lower response is indicated.

Non-androgenic parent	Androgenic parent	Embryogenic anthers (%)	ELS/100AC	P/100AC	P/100EC	GP/100AC	GP/100EC	A/100AC	A/100EC
P10	P2	ns	ns	ns	ns	ns	ns	ns	ns
	P169	ns	ns	ns	ns	ns	ns	ns	ns
P48	P2	ns	ns	ns	ns	ns		ns	ns
	P102	ns	ns	ns	ns	ns	higher*	ns	ns
P133	P2	lowest*	ns	ns	ns	ns	ns	ns	ns
	P102		ns	ns	ns	ns	ns	ns	ns
	P169		ns	ns	ns	ns	ns	ns	ns
Androgenic parent	**Non-androgenic parent**	**Embryogenic anthers (%)**	**ELS/100AC**	**P/100AC**	**P/100EC**	**GP/100AC**	**GP/100EC**	**A/100AC**	**A/100EC**
P2	P10								
	P48								
	P133		higher than P48, P144*				ns	higher than P48, P144*	higher than P144*
	P144				ns		ns		ns
	P175	lowest***	lowest**	lowest**	lowest*	lowest*	lower*	lowest**	lowest*
P102	P48	ns	ns	ns	higher*	ns	higher**	ns	ns
	P133	ns	ns	ns		ns		ns	ns
P169	P10		ns		ns	ns	ns		ns
	P133	higher**	ns	higher*	ns	ns	ns	higher*	ns

A – albinos; AC – anthers cultured; ELS – embryo-like structures; EC – ELS cultured; GP – green plants; P – plants. *** $P \leq 0.001$; ** $P \leq 0.01$; * $P \leq 0.05$; ns – not significant



Supplementary Fig. S4.1 Boxplots of the responses of the evaluated populations to anther culture. The letters above each plot denote significant differences between populations (P ≤ 0.05). **A** – number of albino plants (AP) regenerated per 100 anthers cultured (AC); **B** – number of AP regenerated per 100 embryo-like structures cultured (EC). For graphical reasons, observation 800 from population 11 has been omitted from graph **B**.

8.3 Supplementary information for chapter 5

Table S5.1 Overview of paircross parents and their progeny populations used for phenotypic evaluation of *in vitro* anther culture capacity.

	Parents			**Number of genotypes evaluated in**		
Population	Androgenic		Non-androgenic	2015	2016	2015 and 2016
1	P2	×	P133[1]	49	50	45
2	P2	×	P10[1]	43	-	-
3	P2	×	P48[1]	30	-	-
4	P102	×	P133[1]	25	32	18
6	P102	×	P48[1]	15	21	8
7	P169	×	P133[1]	17	13	7
8	P169	×	P10[1]	20	-	-
10	P2	×	P144[2]	48	-	-
11	P2	×	P175[3]	48	-	-
12	P2	×	P84[2]	11	-	-
15	P102	×	P84[2]	7	-	-
Sum				313	116	78

[1]Many albinos, no green plants; [2]Many embryos, no plants; [3]No embryos, no plants.

Table S5.2 The set of 192 unique (5-10 bp) single restriction enzyme (PstI) genotyping-by-sequencing barcodes and PCR primers used in this study. Barcodes were designed using the Deena Bioinformatics online GBS Barcode Generator (http://www.deenabio.com/nl/services/gbs-adapters) and synthesized by Microsynth (Balgach, Switzerland).

Oligo ID	**Sequence (5' - 3')**
2174689	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGTGTTTGCA
2174690	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAACCATGCA
2174691	ACACTCTTTCCCTACACGACGCTCTTCCGATCTACATATGCA
2174692	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGAAGATGCA

Oligo ID	Sequence (5' - 3')
2174693	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTTCTATGCA
2174694	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAGTCGTGCA
2174695	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTAATGTGCA
2174696	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGTTCATGCA
2174697	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCAGATTGCA
2174698	ACACTCTTTCCCTACACGACGCTCTTCCGATCTATAGGTGCA
2174699	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCGATTTGCA
2174700	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAATGTTGCA
2174701	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTGCATTGCA
2174702	ACACTCTTTCCCTACACGACGCTCTTCCGATCTACAGTATGCA
2174703	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGCTCCATGCA
2174704	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCATGCATGCA
2174705	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGAATCATGCA
2174706	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCCTAAGTGCA
2174707	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTGGCAATGCA
2174708	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGTAGCGTGCA
2174709	ACACTCTTTCCCTACACGACGCTCTTCCGATCTACCTAGTGCA
2174710	ACACTCTTTCCCTACACGACGCTCTTCCGATCTATGACGTGCA
2174711	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCTAGAATGCA
2174712	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGATACGTGCA
2174713	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTCCGAATGCA
2174714	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCAGTGATGCA
2174715	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAGTCCGTGCA
2174716	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGTCCAATGCA
2174717	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCAGGTGTGCA
2174718	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTCACGATGCA
2174719	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTGCAAGTGCA
2174720	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGCGTATGCA
2174721	ACACTCTTTCCCTACACGACGCTCTTCCGATCTATACGGTGCA
2174722	ACACTCTTTCCCTACACGACGCTCTTCCGATCTACTGCTTGCA
2174723	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCGGATATGCA
2174724	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTAGTCTTGCA
2174725	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTCTTGTATGCA
2174726	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCCTGCAATGCA
2174727	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTTATACGTGCA
2174728	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTGCACAATGCA
2174729	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCCGTGCATGCA
2174730	ACACTCTTTCCCTACACGACGCTCTTCCGATCTATACAAGTGCA
2174731	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGTCCGATGCA
2174732	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAATGCCGTGCA
2174733	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGCGTTAATGCA
2174734	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCTCAAGATGCA
2174735	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTAGCCGATGCA
2174736	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGTACGCGTGCA
2174737	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAACGCTATGCA
2174738	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTCTATGGTGCA

Oligo ID	Sequence (5' - 3')
2174739	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGCTATATGCA
2174740	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCAGGTCGTGCA
2174741	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGTGACAATGCA
2174742	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTCGTAGATGCA
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2174782	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTTCGCCGCATGCA
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2174788	TCTTCAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
2174789	TAGAAAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
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2174832	CGCGTACAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
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2174856	TGTGAGCCAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
2174857	TGCGAGGAAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
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	CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT[2]

[1]Common adaptor; [2]PCR primer.

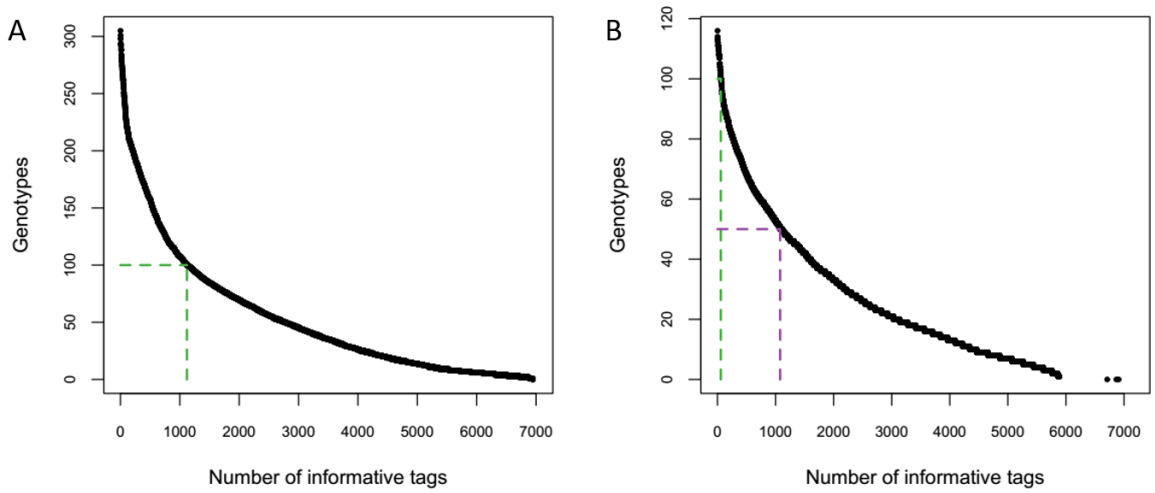

Figure S5.1 Numbers of informative tags per number of genotypes in 2015 (**A**) and 2016 (**B**) with a minor allele frequency of 10%. In this study, a 100 genotype threshold was used for the 2015 data, resulting in 1120 informative tags (green line in graph **A**) and a 50 genotype threshold resulting in 1079 informative tags (purple line in graph **B**) was used for the 2016 data.

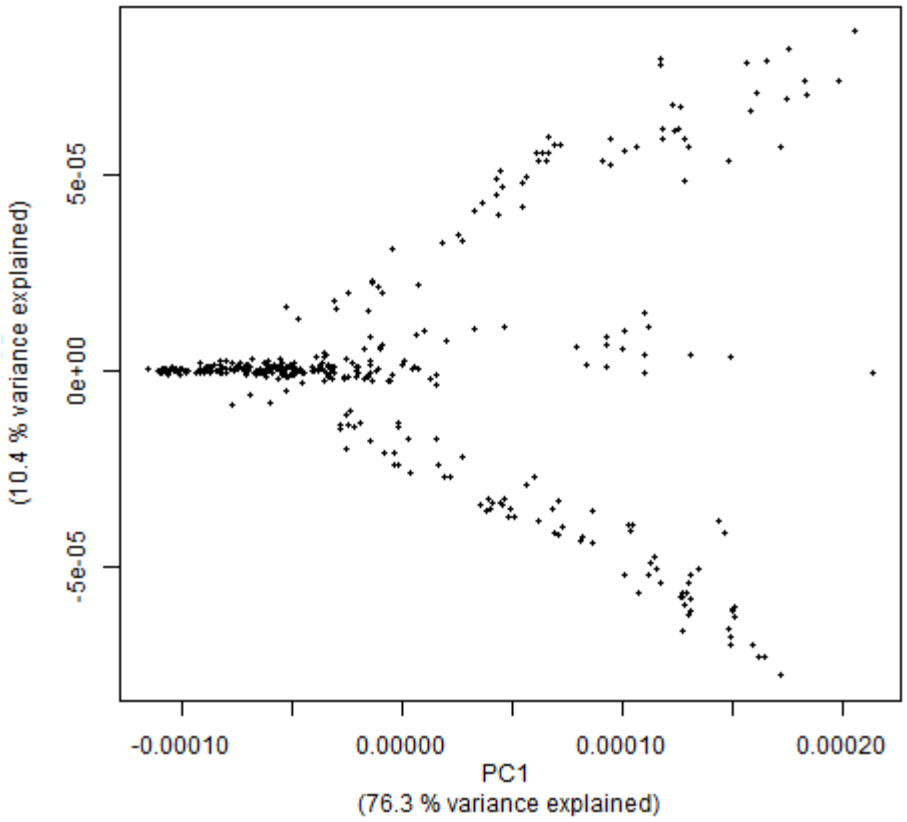

Figure S5.2 The two principal components explaining the greatest variation from a principal component analysis of the genotypic information.

Table S5.3 Gene annotations for each scaffold found to be significantly associated with the response to anther culture.

Scaffold	Scaffold length (kbp)	Start (bp)	End (bp)	Arabidopsis locus	Name(s) or description
60	277.8	7100	73719	AT5G52450	MATE efflux family protein
		36155	38008	AT5G52450	MATE efflux family protein
		41600	42255	AT5G52450	MATE efflux family protein
		114910	119696	AT2G15240	UNC-50 family protein
123	256.4	22922	26802	AT3G02410	ICME-LIKE2, ISOPRENYLCYSTEINE METHYLESTERASE-LIKE 2
		22922	26802	AT5G15860	ATPCME, ICME, ISOPRENYLCYSTEINE METHYLESTERASE, PCME, PRENYLCYSTEINE METHYLESTERASE
		27463	31921	AT3G18040	MAP KINASE 9, MPK9
		197961	200766	AT5G07990	CYP75B1, CYTOCHROME P450 75B1, D501, TRANSPARENT TESTA 7, TT7
		204751	213979	AT5G52450	MATE efflux family protein
127	250.4	38352	41786	AT1G19330	Histone deacetylase complex subunit
		227351	228127	AT1G29860	ATWRKY71, EXB1, EXCESSIVE BRANCHES1, WRKY DNA-BINDING PROTEIN 71, WRKY71
233	212.6	13284	39350	AT5G15070	ARABIDOPSIS HOMOLOG OF YEAST VIP1 2, ATVIP2, VIH1, VIP HOMOLOG 1, VIP1 HOMOLOG 2, VIP2
		123380	130183	AT5G62670	AHA11, H(+)-ATPASE 11, HA11
		131144	134955	AT1G27150	Tetratricopeptide repeat (TPR)-like superfamily protein
		199043	204791	AT3G05545	RING/U-box superfamily protein
813	154	34360	44241	AT1G33290	P-loop containing nucleoside triphosphate hydrolases superfamily protein
		98603	105466	AT5G14420	RGLG2, RING DOMAIN LIGASE2
		149380	153333	AT1G47830	SNARE-like superfamily protein
815	148.4	7582	22714	AT2G20330	Transducin/WD40 repeat-like superfamily protein
		23376	32394	AT4G21470	ATFMN/FHY, FMN/FHY, RIBOFLAVIN KINASE/FMN HYDROLASE
		58149	62391	AT2G01120	ATORC4, ORC4, ORIGIN RECOGNITION COMPLEX SUBUNIT 4
		139557	146531	AT5G43920	Transducin/WD40 repeat-like superfamily protein

Scaffold	Scaffold length (kbp)	Start (bp)	End (bp)	Arabidopsis locus	Name(s) or description
1607	115.8	28155	33804	AT5G57140	ATPAP28, PAP28, PURPLE ACID PHOSPHATASE 28
		44535	50663	AT2G27100	SE, SERRATE
		89740	92906	AT3G07040	RESISTANCE TO PSEUDOMONAS SYRINGAE 3, RPM1, RPS3
		100317	104480	AT3G07040	RESISTANCE TO PSEUDOMONAS SYRINGAE 3, RPM1, RPS3
1669	116.6	36273	49336	AT3G63520	ATCCD1, ATNCED1, CAROTENOID CLEAVAGE DIOXYGENASE 1, CCD1, NCED1
2075	106.6	6177	6430	AT1G70680	Caleosin-related family protein
		7022	9674	AT1G70670	ARABIDOPSIS THALIANA CALEOSIN 4, ATCLO4, CALEOSIN 4, CLO4, PEROXYGENASE 4, PXG4
2554	103	2585	8463	AT2G45260	Myosin-4 protein (DUF641)
		46779	53204	AT5G35210	PHD TYPE TRANSCRIPTION FACTOR WITH TRANSMEMBRANE DOMAINS, PTM
3194	88.8	21212	27087	AT3G46820	TOPP5, TYPE ONE SERINE/THREONINE PROTEIN PHOSPHATASE 5
		36911	38396	AT5G64700	UMAMIT21, USUALLY MULTIPLE ACIDS MOVE IN AND OUT TRANSPORTERS 21
		43210	51417	AT2G47580	SPLICEOSOMAL PROTEIN U1A, U1A
		51670	56416	AT3G52860	MED28, MEDIATOR28
3723	81.3	37204	45974	AT1G63810	Nucleolar protein
		80302	80933	AT4G23160	CRK8, CYSTEINE-RICH RLK (RECEPTOR-LIKE PROTEIN KINASE) 8
4385	80.8	20744	21199	AT5G26880	AGAMOUS-LIKE 26, AGL26
		35922	58157	AT3G23640	HETEROGLYCAN GLUCOSIDASE 1, HGL1
		67663	70662	AT4G15240	Glycosyltransferase (DUF604)
		71970	75561	AT3G20740	FERTILIZATION-INDEPENDENT ENDOSPERM 1, FIE, FIE1, FIS3
6186	59.2	35206	36492	AT1G70090	GALACTURONOSYLTRANSFERASE-LIKE 9, GATL9, GLUCOSYL TRANSFERASE FAMILY 8, LGT8
		47809	54233	AT1G12000	Phosphofructokinase family protein

Scaffold	Scaffold length (kbp)	Start (bp)	End (bp)	Arabidopsis locus	Name(s) or description
6436	57	45334	47951	AT4G13330	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein
		48789	50285	AT5G20190	Tetratricopeptide repeat (TPR)-like superfamily protein
		51151	57039	AT5G43960	Nuclear transport factor 2 (NTF2) family protein with RNA binding (RRM-RBD-RNP motifs) domain-containing protein
7045	53.2	1236	5326	AT4G38180	FAR1-RELATED SEQUENCE 5, FRS5
		6122	14989	AT2G23380	CLF, CURLY LEAF, ICU1, INCURVATA 1, SDG1, SET1, SETDOMAIN 1, SETDOMAIN GROUP 1
		39822	46005	AT5G52010	C2H2-like zinc finger protein
		39823	45693	AT1G28600	GDSL-motif esterase/acyltransferase/lipase
8920	44.9	2729	10974	AT1G75850	LAZ4, VPS35 HOMOLOG B, VPS35B
15142	24.8	16759	22240	AT5G08560	ATWDR26, WD-40 REPEAT 26, WDR26
16597	14.2	7880	8705	AT5G54960	PDC2, PYRUVATE DECARBOXYLASE-2

9. Acknowledgements

First of all, I would like to express my gratitude to my supervisor, Bruno, who gave me the opportunity to try my hand at science. We have had to get used to one another and there have been some bumps in the road, but you have always made time for me when I needed support. I appreciate the trust and freedom you have given me and have very much enjoyed writing papers together. Thank you.

I would also like to thank my colleagues in the Molecular Plant Breeding group. Steven, your encouragement while I was trying to teach myself programming in R has been so very motivating and I would never have been able to analyse my sequencing data without your help. Tim, thank you for calming me down during library preparation in the lab and for patiently answering all the questions about genomics I have bombarded you with over the years. Chloé, Michelle, Daniel, Mike, Lea, Verena, Roland and everyone else, I am grateful to have worked with all of you. And of course my collaborators at DLF, Niels, Kirsten and all the others, thank you for welcoming me so warmly during my visit as a freshly minted doctoral student.

What an undertaking it has been, these three years of science! I would certainly never have made it through in one piece without the unconditional love and support of my family: my parents, my sister and her three wonderful children. Thank you, for everything.

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Guillaume, we were in this together, somehow, and you know what that has meant to me.

10. Curriculum Vitae

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Personal details

Date and birth place:	May 3rd 1989, Voorburg, the Netherlands
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E-mail:	rachel.begheyn@usys.ethz.ch or rachel.begheyn@gmail.com

Expertise and skills

Expertise:	Plant breeding, plant biotechnology, plant genetics, plant tissue culture, doubled haploids, scientific writing, literature research and statistics
Language skills:	Dutch (native), English (full professional ability), German (B2), Spanish (A2)
Computer skills:	MS Office 2010, R and R Studio

Education and research experience

April 2014 – September 2017	Doctoral candidate at ETH Zürich, Switzerland Supervisor: Prof. Bruno Studer (Molecular Plant Breeding) Collaborator: DLF A/S, Denmark Thesis title: Efficient doubled haploid production in forage grasses
February 2014 – April 2017	Ph.D. project proposal entitled "Efficient doubled haploid production in forage grasses" with Prof. Bruno Studer was funded by ETH Zurich (Research Grant ETH-34 14-1; CHF 243'400)
September 2011 – October 2013	MSc Plant Sciences at Wageningen University, The Netherlands Specialization in Plant Breeding and Genetic Resources Master thesis title: Interspecific crosses in leek and onion to introgress resistance to a globally significant pest: thrips Internship 1: Heterosis in tomato at Enza Zaden BV in Almería, Spain Internship 2: Cotton seed production system, Louis Bolk Institute, Lira, Uganda
September 2008 – August 2011	BSc Plant Sciences at Wageningen University, The Netherlands Specialization in Plants and Human Health

Publications

R.F. Begheyn, S. A. Yates, T. Sykes and B. Studer, "Genetic loci governing androgenic capacity in perennial ryegrass (*Lolium perenne* L.)", *in preparation*, **2017**.

R.F. Begheyn, N. Roulund, K. Vangsgaard, D. Kopecký and B. Studer, "Inheritance patterns of the response to *in vitro* doubled haploid induction in perennial ryegrass (*Lolium perenne* L.)". Plant Cell, Tissue and Organ Culture (PCTOC), vol. 130, iss. 3, pp. 667-679, **2017**.

R.F. Begheyn, T. Lübberstedt, and B. Studer, "Haploid and doubled haploid techniques in perennial ryegrass (*Lolium perenne* L.) to advance research and breeding," Agronomy, vol. 6, iss. 4, paper number 60, **2016**.

Selected conference papers and presentations

R.F. Begheyn, N. Roulund, K. Vangsgaard, D. Kopecký and B. Studer, "Inheritance patterns of the response to *in vitro* doubled haploid induction in perennial ryegrass (*Lolium perenne* L.)". Presentation at the 15th Plant and Animal Genome (PAG) conference, January 14-17th **2017**, San Diego, CA, USA.

R.F. Begheyn, "Doubled haploid induction in perennial ryegrass". Presentation at the 5th Minisymposium Molecular Forage Crop Breeding, May 2nd **2016**, ETH Research Station, Lindau-Eschikon, Switzerland.

R.F. Begheyn, K. Vangsgaard, N. Roulund and B. Studer, "Efficient doubled haploid production in perennial ryegrass (*Lolium perenne* L.)," in Breeding in a world of scarcity: Proceedings of the 2015 Meeting of the Section "Forage Crops and Amenity Grasses" of EUCARPIA, Ghent, Belgium, Springer International Publishing, **2016**, pp. 151–155. Mini-paper presented at the conference and published in the proceedings.

Selected extracurricular activities

2016	Member of the organization committee of the December 1st 2016 Zurich-Basel Plant Sciences Center Symposium "Plants and human health – from research to application".
2010 – 2012	Student member of Study Program Committee of BSc and MSc Plant Sciences and MSc Plant biotechnology, Wageningen University, The Netherlands.
2010 – 2011	Board member of Semper Florens, the study association of the BSc and MSc Plant Sciences at Wageningen University, The Netherlands.