Genetic and genomic analysis of polyploid *Chrysanthemum* hybrids with emphasis on shoot branching

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Abstract

Chrysanthemum indicum hybrids are among the most important ornamentals worldwide and their success is based on their abundant phenotypic diversity. Especially, shoot branching is crucial due to their use as cut flowers and potted plants. Therefore, shoot architecture is manually shaped by disbudding for cut flower production, which is time consuming and costly. Although, breeders are always in need of and searching for innovations, the genetics and regulation of shoot branching in chrysanthemums has been only analysed to a limited extent.

In this study two different chrysanthemum populations, a collection of varieties and a biparental F1 population, have been characterised for shoot branching and other ornamental traits. The analysis of AFLP® markers revealed marker-trait associations for the genotype collection using a genome-wide association study and marker-trait associations for the population by applying a single locus analysis. In addition to focus on shoot branching a candidate gene approach for strigolactone (SL) pathway genes was used. With this approach marker alleles were identified that were significantly associated with bud outgrowth in both populations. These polymorphisms described a large proportion of the variation in shoot branching in these populations. Moreover, the yet unknown *CCD*7/*MAX*3 gene of chrysanthemum was characterised. Its expression and the expression of other SL pathway genes was analysed in different weak and strong branched genotypes of the F1 population. Beside the application of the SL analogue CISA-1 inhibited shoot branching. Thus, these results highlight the importance of SL in the regulation of shoot branching in the chrysanthemum; though, other yet unknown factors are also likely involved.

In addition, the applied molecular markers provided insight into the genetics and genomics of chrysanthemums. For instance, the phylogeny of the collection of varieties could not be easily revealed indicating a complex breeding history involving repeated backcrosses, and the exchange of genotypes between breeders. Based on molecular marker analyses, a polysomic inheritance was detected in chrysanthemums, as is characteristic of an autopolyploid. Hence, chrysanthemums should be considered as segmental allohexaploids.

Keywords: molecular marker, polysomic inheritance, strigolactone

Zusammenfassung

Die Hybriden von *Chrysanthemum indicum* gehören zu den weltweit bedeutendsten Zierpflanzen. Ihr Erfolg gründet auf der Mannigfaltigkeit an Phänotypen, wobei die Verzweigungsneigung besonders wichtig ist. *C. indicum* Hybriden werden sowohl als Schnitt- als auch als Topfpflanzen verkauft, was eine manuelle Bearbeitung des Pflanzenbaus erfordert. Dies verursacht hohe Kosten und daher wäre eine genetische Kontrolle der Sprossverzweigung bei Chrysanthemen wünschenswert.

In der vorliegenden Arbeit wurden zwei unterschiedliche Chrysanthemenpopulationen, eine Sammlung von Sorten und eine biparentale F1 Population, hinsichtlich ihrer Sprossverzweigung und weiterer, bedeutender Eigenschaften charakterisiert. Diese Daten wurden mit den Spaltungsmustern von AFLP® Markern verrechnet. Dadurch konnten für beide Populationen Marker-Merkmals-Assoziationen identifiziert werden. Strigolakton (SL) wurde vor kurzem als Phytohormon charakterisiert, das den Auswuchs von Seitentrieben reguliert. Daher wurden Kandidatengene des SL-Synthesewegs hinsichtlich informativer DNA Unterschiede untersucht. Für alle Gene waren solche DNA Unterschiede mit der Verzweigungsneigung in beiden Populationen assoziiert und auf diese Polymorphismen konnte ein großer Teil der gefundenen Varianz für das Merkmal zurückgeführt werden. Zusätzlich wurde das bisher unbekannte *CCD*7/*MAX*3 Gen der Chrysantheme isoliert und die Expression dieses Gens sowie der anderen Kandidatengene in wenig und stark verzweigenden Genotypen detektiert. Darüber hinaus unterdrückte das SL-Analogon CISA-1 den Austrieb. Somit stützen diese Untersuchungen die Bedeutung des Phytohormons SL bei der Regulation der Sprossverzweigung. Allerdings scheinen weitere in dieser Arbeit noch nicht identifizierte Faktoren ebenfalls eine Rolle zu spielen.

Die Analyse der molekularen Marker lieferte Erkenntnisse zur Genetik und Genomorganisation bei Chrysanthemen. Zum einen waren die untersuchten Sorten mit phylogenetischen Methoden kaum zu unterscheiden, was auf einen komplizierten Entstehungsprozess genetisch eng verwandter Genotypen hindeutet. Zum anderen weist die Analyse der molekularen Marker eine polysome Vererbung für die Chrysanthemen nach. Dies ist typisch für Autopolyploide und daher sollten Chrysanthemen als segmental Allohexaploide und nicht, wie bisher, als Allopolyploide betrachtet werden.

Schlagwörter: molekularer Marker, polysome Vererbung, Strigolakton

Contents

Abbreviations

AA	amino acid
AFLP®	amplified fragment length polymorphism
AM	axillary meristem
ANOVA	analysis of variance
APS	ammonium persulfate
CCD	carotenoid cleavage dioxygenase
CK	cytokinin
EDTA	ethylenediaminetetraacetic acid
GWAS	genome wide association study
MAX	more axillary branching
MD	multi-dose
MLM	mixed linear model
PAT	polar auxin transport
QTL	quantitative trait locus
SAM	shoot apical meristem
SD	single-dose
SL	strigolactone
SSCP	single-strand conformation polymorphism
SSR	microsatellite
TBE	tris/borate/EDTA buffer
TEMED	tetramethylethylenediamine

1 General introduction

This study aimed at generating details of genetic and genomic processes in chrysanthemum hybrids (*Chrysanthemum indicum* hybrids) using molecular markers. In this context, particular emphasis was put on the regulation of shoot branching. The resulting major objectives of the study are formulated at the end of this chapter.

This chapter provides background information to the following main aspects considered in this thesis:

i) the developmental history and the horticultural impact of chrysanthemum hybrids (*Chrysanthemum indicum* hybrids),
ii) the concepts to identify marker-trait associations and
iii) the current model to control shoot branching.

1.1 The ornamental chrysanthemum

Chrysanthemums are considered to be old ornamental crops with a long breeding history, especially in China and Japan. In China the cultivation and breeding of chrysanthemums already started more than 2000 years ago and in Japan the significance of chrysanthemums is addressed by its adoption as the official seal of the Emperor (Vogelmann, 1969, S. 22).

To Europe chrysanthemums were introduced first as the so called garden varieties via the Netherlands from China probably at the end of the 17th century (Anderson, 2006). These Chinese varieties were characterised by small, ball-formed flowers and by late flowering.

The first European breeding centres were France and England. However, after 1860 the European breeding programmes sustainably changed only after the introduction of well established Japanese varieties. The newly introduced varieties showed a great diversity in flower type and colour and were large-flowered. These characteristics were crucial for the success of their ornamental value in the Western world (Vogelmann, 1969, S. 21f).

In the following, chrysanthemums are characterised taxonomically and morphologically. Their huge phenotypic diversity is the basis for their economic importance as horticultural crops.

1.1.1 The taxonomy and the geographical distribution of *Chrysanthemum* species

Chrysanthemum indicum hybrids belong to the family of *Asteraceae*, which is one of the biggest families of the angiosperms including other important crops, such as sunflower (*Helianthus annuus*), lettuce (*Lactuca sativa*), gerbera (*Gerbera* L.) or garden dahlia (*Dahlia variabilis*). Within the *Asteraceae* chrysanthemum hybrids are grouped in the genus *Chrysanthemum* with 40 species. The word chrysanthemum is derived from the Greek words 'chrysos' (gold) and 'anthemon' (flower) meaning golden flower (Morton, 1891).

The chrysanthemum species are distributed in the temperate climate zone to the subtropics of the northern hemisphere with Eastern Asia as main area (Anderson, 2006). They grow only seasonal or perennial as herbaceous or as semi-shrubby plants in the grasslands as well as on stony soil (Anderson, 2006). The most important domesticated species, such as *C. x morifolium*, C*. indicum*, *C. japonicum* and *C. weyrichii*, were grouped to the genus *Dendranthema* (Anderson, 2006). However, this naming of the genus was contentious and subsequently changed to the defining species *Chrysanthemum indicum* by a rule of the International Code of Botanical Nomenclature in 1999. The recent garden forms are known as *C. indicum* hybrids.

1.1.2 The morphology of cultivated chrysanthemums

Chrysanthemums display a huge phenotypic diversity in growth habit, flower colour, flower shape and leaf form. However, there are some characters common to all chrysanthemums. The whole generative shoot of a chrysanthemum is a cyme with multiple inflorescences, of which the oldest terminates the main shoot (Cockshull, 1985). The inflorescences consist of central hermaphrodite disc florets (pistil and stamen) and marginal female ray florets (pistils) with inferior ovaries (Cockshull, 1985).

Modifications of the basic flower form are common and cultivars are classified by their flower type. The flower types are (see Figure 1.1a): singles or half-filled (1-5 rows ray florets), filled, spoon types (tubular ray florets with ray floret tips flattened like a spoon), spider types (ray florets are long and quilled, hooked and drooping), pompons (tubular ray florets and no disc florets visible), ball-formed (petal tips curved inward) or reflexed (opposite of incurved), decoratives (outer ray florets longer than centre ones and disk florets hidden) and anemones (centre disc florets tubes elongated and coloured). Since the 1930th there were mainly late flowering varieties available, but then especially English early flowering varieties were introduced to the Western markets (Anderson, 2006).

Furthermore, different plant habits constitute the commercial greenhouse and garden chrysanthemums (Anderson, 2006). These are (see Figure 1.1b for some examples): upright, lilliput, cushion, large shrub and wave (ground-cover plants).

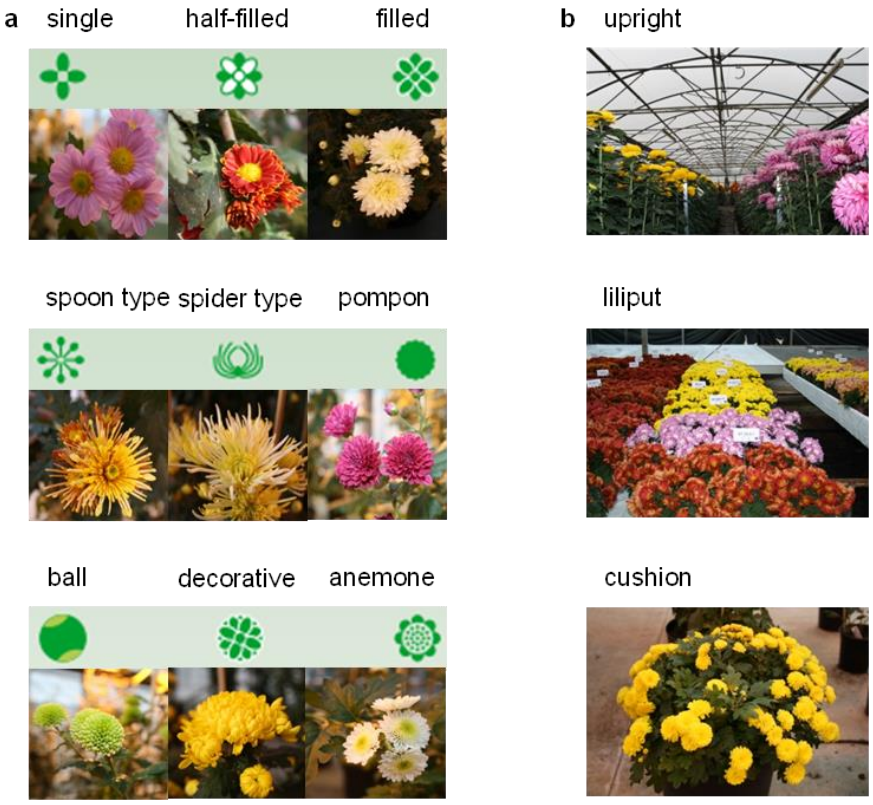

Figure 1.1: Cultivated chrysanthemums are classified by their flower type (a, modified after Brandkamp Chrysanthemenkatalog 2010) and their plant habit (b).

1.1.3 The horticultural impact of chrysanthemums

The abundant diversity in flower type, colour and plant architecture of cultivated chrysanthemums is the basis for its ornamental value (Zhang *et al.*, 2010). There are more than 1000 protected varieties registered at the community plant variety office (CPVO; http://www.cpvoextranet.cpvo.europa.eu/WD150AWP/WD150AWP.exe/CONNECT/ClientExtranet). The chrysanthemums rank second in terms of their ornamental market value in the world, only surpassed by roses (Huylenbroeck, 2010). They are produced as cut flowers, potted plants, bedding and balcony plants throughout the year. For instance, in 2011 the assumed expense based on consumer studies was 133 million € and they were ranked among the top 10 sold cut flowers and potted plants in Germany (Gärtnerbörse 04/2012).

The breeding of new chrysanthemum varieties mainly focuses on the habit of the plant and floral traits. The desired habit is derived from the intended utilization of the cultivar as cut flowers or potted plants. Thus, the outgrowth of side shoots is an important aspect in the breeding of new varieties. For cushions and large shrubs strong branching is required, whereas decorative cut flowers are produced as single stems. Therefore, side shoots are removed manually during the cultivation of cut chrysanthemums. Hence, the breeding objectives for shoot branching might be antagonistic due to the intended use of the plant.

Furthermore, the ornamental market is in permanent need of new flower types and colours. Breeders put a lot of effort into the improvement of the inflorescence because this trait is most important for the ornamental value of a chrysanthemum plant. Nevertheless, breeding for other horticultural traits, such as tolerance towards abiotic stresses (e. g., cold or drought) or resistance against biotic stresses (e. g., pests and diseases), are considered as well.

New cultivars are primarily generated in two different ways. They can either be established by the selection of F1 hybrids resulting from biparental crosses or by obtaining "sports", mainly through induced mutation by irradiation of a single interesting cultivar. Moreover, transgenic strategies bear the potential for the introduction of foreign or beneficial genes into chrysanthemums (recently reviewed by Teixeira da Silva *et al.*, 2013).

Chrysanthemums are propagated vegetatively via cuttings and are mainly cultivated in greenhouses under controlled conditions. Vegetative propagation has the advantage that all cuttings are identical to the parental plant. Important centres of production include China (2,150 million stems in 2010), Japan (1,950 million stems in 2009), Vietnam (600 million stems in 2009), the Netherlands, South Korea, Malaysia, Kenya, Colombia and Brazil (Teixeira da Silva *et al.*, 2013). In Germany 69,000 cut chrysanthemums and 20 million potted plants were produced in 2012 (https://www.destatis.de/DE/ZahlenFakten/Wirtschaftsbereiche/LandForstwirtschaftFischerei/ObstGemueseGartenbau/Tabellen/Zierpflanzenanbau.html). In addition to its ornamental value, chrysanthemums are also cultivated for pharmaceutical purposes, especially in China.

1.1.4 Genetic and genomic studies of chrysanthemums

As chrysanthemums are of great economic importance, an increasing number of research projects were conducted in recent years. For the year 2012 the "Web of Science" (http://thomsonreuters.com/web-of-science/) lists more than 200 publications dealing with *Chrysanthemum* for instance. These papers concentrate on plant protection, valuable metabolites, plant physiology and biotechnological approaches, such as tissue culture or genetic transformation. Although the first cytological analyses were already conducted more than 50 years ago (Dowrick, 1952; Dowrick, 1953), there are only a few publications about the genetics of chrysanthemum available

Based on these studies and their evolutionary origin as the result of natural hybridisations between different species such as *C. indicum* L., *C. morifolium* and others as for example *C. vestitum* or *C. lavandulifolium* (Vogelmann, 1969, S. 10; Dai *et al.*, 1998; Yang *et al.*, 2006) chrysanthemums are believed to be allohexaploid ($2n = 6X = 54$), although aneuploidy is a common phenomena and the chromosome number of 54 can vary between different cultivars (Dowrick, 1953; Roxas *et al.*, 1995, Li *et al.*, 2011). In accordance with their polyploid genome structure chrysanthemums have a large genome with a 1C-value of approximately 9.6 pg (http://data.kew.org/cvalues/). The classification of chrysanthemums as allopolyploid is supported by the high frequency of bivalents in meiosis in different

polyploid *Chrysanthemum* accessions (Dowrick, 1953; Watanabe, 1977; Li *et al.*, 2011). However, a low frequency of multivalents was also observed in meiosis in cytological studies in chrysanthemum (Dowrick, 1953; Chen F *et al.*, 2009; Li *et al.*, 2011).

In contrast to most cytological results supporting the hypothesis of allopolyploidy in chrysanthemum, Langton (1989) detected a hexasomic inheritance for carotenoid pigmentation. This would indicate a polysomic inheritance for chrysanthemums, as it is characteristic for autopolyploids. It is therefore not clear whether most characters are inherited in a disomic (bivalent formation and interspecific hybridization) or hexasomic manner. Hence, it is questionable, whether cytological methods are sufficient to determine the type of ploidy because their results do not directly and reliably infer from the inheritance. Currently, there are more informative and reliable methods available that are based on molecular marker analyses, since molecular markers directly detect the mechanisms of inheritance in a genome.

Wu *et al.* (1992) described the usefulness of single-dose (SD) molecular markers to determine the type of ploidy. SD markers are characterised by only one dominant marker allele at a single locus and can be distinguished from multi-dose markers (MD) by identifying the means of the corresponding recombination frequencies (Mather, 1957). Da Silva *et al.* (1993) determined the theoretical ratios of SD to MD markers for allo- and autopolyploids, which might indicate the ploidy type of an organism. Furthermore, Wu *et al.* (1992) showed that allopolyploids are characterised by a higher frequency of linkages in repulsion than autopolyploids. By calculating the ratio of markers in coupling to those in repulsion, it is possible to distinguish allopolyploidy (ratio of 1:1) from autopolyploidy (ratio of 1:0 for polyploids above tetraploidy). Additionally, the banding patterns of sequence specific codominant single locus markers, such as microsatellites (SSRs), reflect the distributions of the homologous and homeologous chromosomes within the progeny. Thus, this type of marker is informative in determining the pairing of the chromosomes because SSR marker alleles are inherited in all possible combinations under polysomic inheritance. On the contrary under disomic inheritance pairs of alleles are never inherited together.

Genetic analyses are further complicated by the outcrossing nature of chrysanthemums due to a polygenic, sporophytic self-incompatibility system (Drewlow *et al.*, 1973; Anderson *et al.*, 1992). Hence, many crosses between related or unrelated cultivars are not successful and inbred lines are seldom because they suffer from inbreeding depression (Anderson *et al.*, 1992). By analysing the genetic diversity within the breeding germplasm, it could be possible to select favourable parents for experimental segregating populations. In both of these the degree of phenotypic and marker polymorphism is maximised with respect to unselected parental combinations. This would significantly facilitate the genetic analyses of many important ornamental traits in chrysanthemums and improve accordingly the targeted breeding strategies supported by molecular markers.

As a strictly outcrossing species, chrysanthemums are highly heterozygous and the genetic analysis of many traits is additionally impaired by the complex, polyploid genome structure. With the rise of molecular markers over recent years this inadequacy might have overcome, although markers (e. g., random fragment length polymorphism [RFLP], random amplified polymorphic DNA [RAPD], or inter-simple sequence repeats [ISSR]) have been mainly used to identify sports and cultivars in chrysanthemum (Wolff *et al.*, 1995; Wolff, 1996; Huang *et al.*, 2000). Nevertheless, Zhang *et al.* (2010) calculated and improved a genetic map in chrysanthemum and located quantitative trait loci (QTLs) for inflorescence-related (Zhang *et al.*, 2011a) and flowering traits (Zhang *et al.*, 2011b); though, the marker saturation of the map is still limited and the identified markers for these traits are not easily transferable to another genetic background of chrysanthemums.

Recently, the costs for DNA sequencing approaches decreased rapidly. Thus, the near future holds for available resources of high marker density based on next-generation sequencing techniques. These techniques were already used to generate expressed sequence tag (EST)-library (Chen S *et al.*, 2009) and a whole genome expression profile under dehydration stress (Xu *et al.*, 2013) for chrysanthemum. These sequence information are useful to identify repeated sequence motifs, which have been integrated to the Chrysanthemum Transcriptome Database (http://www.icugi.org/chrysanthemum), for the development of microsatellite markers (SSRs) or to identify single nucleotide

polymorphisms (SNPs). Especially, with the help of SNPs it is possible to achieve a high marker density, although the analysis of the marker polymorphisms of SNPs is not trivial in a hexaploid genome due to the high degree of genetic redundancy.

The identification of additional or other loci for candidate genes using molecular markers for important horticultural traits, such as flower diameter or shoot branching, could improve the breeding process in chrysanthemum. Consequently, this study aims at the identification of associations between markers and important traits. Some of the concepts are introduced in the following section.

1.2 The identification of loci for candidate genes

The identification of genetic loci contributing to important traits is a prerequisite for quality breeding because phenotypical and statistical analyses during the process of selection are often time-consuming and difficult. Consequently, the application of genetic markers that enable genotyping at an early stage, independently from changing environmental conditions, could make the selection process time- and cost-effective. Furthermore, genetic markers might be used diagnostically to predict the presence of certain genes that are responsible for a trait (e. g., resistance genes against pathogens or genes for flower pigmentation). Thus, they might help to understand the underlying genetics of complex physiological processes. There are different strategies available to identify loci for candidate genes, such as the detection of quantitative trait loci (QTLs) in combination with linkage mapping, genome wide association studies and candidate gene approaches. These strategies are introduced in the following subsections.

1.2.1 The principle of QTL mapping

Many important ornamental traits (e. g., control of flower formation, leaflet morphology or some forms of disease resistance) are controlled by multiple genes and are influenced by the environment. Therefore, these traits are termed quantitative traits. A genetic region that contains one or several genes associated with a quantitative trait, is called a QTL (Collard *et al.*, 2005). Such a QTL can be localized in the genome for example by genetic linkage mapping based on the recombination frequencies of molecular markers.

Typically populations resulting from biparental crosses with contrasting genotypes differing in one or more traits of interest are used for the QTL mapping. The larger the population size, the higher could be the resolution of the mapping approach. Basis for the detection of an association between a marker and a QTL is the occurrence of linkage disequilibrium (LD) between them. This means the non-random association of alleles at two or more loci that descend from a single, common ancestral chromosome. The simplest approach for detecting a QTL is to analyse the data using one marker at a time by applying statistical tests (Tanksley, 1993). For such approaches, no complete linkage map is needed. In contrast, by using interval mapping the sets of linked markers and the intervals between them are analysed simultaneously with regard to their effects on a trait (Tanksley, 1993). By using linked markers for the analysis, it is possible to compensate for recombination between the markers and the QTL. This increases the probability of statistically detecting the QTL and also to provide an unbiased estimate of the QTL effect on the character (Tanksley, 1993).

The main advantages of QTL mapping are the possibility to detect even loci that contribute only to a small extent to the trait and to identify genotype-by-environment interactions by testing several environments (Des Marais *et al.*, 2013). Meanwhile, several QTL mapping studies are available for many important agricultural crops. These approaches have been used to identify countless QTLs and currently epistatic interactions between different QTLs and environmental effects are characterised to further investigate the genetic control of certain traits (Ma *et al.*, 2006; Kumar *et al.*, 2013). The next step in the development is to combine expression studies with QTL mapping to colocalise differentially expressed genes with trait-related QTLs (Des Marais *et al.*, 2013). However, there are only a few mapping studies for ornamentals (e. g., Han *et al.*, 2002; Spiller *et al.*, 2010; Zhang *et al.*, 2011a; or Shahin *et al.*, 2011) and association studies became meanwhile more favoured than QTL analysis.

1.2.2 The principle of association studies

In contrast to QTL mapping, in association studies a collection of genotypes with unknown ancestry is used to examine the linked inheritance of QTLs with adjacent molecular markers. These genotypes could be a collection of wild species, landraces, or cultivars. This allows evaluating a broader genetic and phenotypic variation because the approach is not limited to the marker and trait loci differing between two parents (Kraakman *et al.*, 2006).

In simple terms, in an association study the allele frequencies of a marker set are compared between a group of unrelated individuals with the desired trait and a group of unrelated individuals without the trait. If a marker is linked with the phenotype, it will display a significant different allele frequency than the unlinked markers (Pritchard *et al.*, 2000). Hence, LD has to occur between a marker polymorphism and the trait in the plant germplasm. Especially, low levels of LD between a molecular marker and the causes for phenotypic variation occur in unstructured populations because a high number of recombination events accumulated in their breeding history. Thus, associations will only be present between QTLs and markers that are tightly linked to it. This results in an increase of the mapping resolution (Flint-Garcia *et al.*, 2003), if sufficient molecular markers and segregating genotypes are available. Factors leading to a decrease in LD are outcrossing, high recombination rate, high mutation rate or gene conversion (Sorkheh *et al.*, 2008). On the contrary a small population size, inbreeding, population admixture, genetic drift or epistasis for instance lead to an increase (Sorkheh *et al.*, 2008).

To avoid false positive associations it is necessary to investigate the structure of a chosen population. Since there are distinct, related subgroups, many markers will appear to be correlated with the trait. But in reality they are only capturing the genetic relatedness among individuals (Myles *et al.*, 2009). Thus, it is necessary to correct for the genetic relatedness in association studies. Such a population structure could be detected through distance-based or model-based methods. Distance-based methods correspond to phylogenetic trees and are rarely used. In contrast model-based methods, such as maximum-likelihood or Bayesian methods, assume that the population structure has similar effects on all loci. In consequence,

a great number of independent marker loci are used to detect intrinsic populations and to correct for them (Pritchard and Rosenberg, 1999). Furthermore, it is possible to generate a kinship matrix (K) by using independent marker loci to assist in estimating and correcting genetic relatedness of individuals (Myles *et al.*, 2009). Such a correction of the population structure and kinship allows the mixed linear model (MLM) of the computer software *structure* 2.3.2.1 (Falush *et al.*, 2003; Falush *et al.*, 2007; Hubisz *et al.*, 2009) providing a powerful tool to perform association studies.

Two different approaches are currently used to identify QTLs using association studies: genome wide association studies (GWAS) and candidate gene approaches. In the following both of them are discussed in more detail.

Genome wide association studies

The idea behind GWAS is to screen an entire genome for marker-trait associations. Hence, a high number of unlinked and putatively neutral molecular markers are needed (Hall *et al.*, 2010), which cover all chromosomes and most of the genome under research. The number of markers is dependent on the investigated species, its genome size and the decay of LD (Rafalski, 2010). Suitable marker types for GWAS are amplified fragment length polymorphism (AFLP®) due to their numerous occurrence throughout the genome, microsatellites (SSR) and single nucleotide polymorphisms (SNPs). Especially the latter, as sequencing costs are rapidly decreasing and high numbers of SNP markers could be developed. Thus, SNP markers that detect polymorphisms in large numbers, become available and will be of interest even for non-model plants. SNPs have a high genome density and are amenable to high-throughput genotyping in multiplex or microarray format (Syvanen 2001; Syvanen, 2005). However, the genetic resolution of any mapping methodology ultimately depends on the frequency of recombination in the experimental population, as measured by the rate of the decay of LD (Rafalski, 2010).

GWAS have been first applied in human genetics. In plants the first association study was conducted in sea beet (*Beta vulgaris* ssp. *maritima*), which is a wild relative of sugar beet (Hansen *et al.*, 2001). Currently, they become very popular in plant sciences and breeding, as high-throughput genotyping techniques are

available. However, thorough phenotyping of the traits of interest is becoming the (cost) limiting factor in GWAS.

Candidate gene approaches

In contrast to GWAS a candidate gene approach is hypothesis driven. It raises the question, if there is a correlation between DNA polymorphisms in a specific gene and the trait of interest. However, this requires the previous knowledge of casual genes and biochemical pathways controlling a certain trait (Rafalski, 2010). Unfortunately, many other informative genes or loci might not be detected (Rafalski, 2010). Such candidate genes are often initially identified by loss of function mutations. So, it is not clear how well DNA polymorphisms of a candidate gene describe the variation underlying a quantitative trait in a 'natural' population (Hall *et al.*, 2010).

Yet, many candidate gene association studies, such as in barley (*Hordeum vulgare*; Stracke *et al.*, 2009), potato (*Solanum tuberosum*; D'hoop *et al.*, 2008) or sugar beet (*Beta vulgaris*; Stich *et al.*, 2008), were successful in identifying marker-trait associations using tens to hundreds markers in mapping populations of a few hundred individuals (Hall *et al.*, 2010).

In addition to the analysis of DNA polymorphisms candidate genes might also provide valuable information on their molecular level. For instance, the expression of a gene might vary between plants differing in a phenotypic trait. Furthermore, biotechnological approaches, aiming to specify or knock-out a known function of a candidate gene, are valuable sources to generate new varieties with desired traits (Teixeira da Silva *et al.*, 2013).

Therefore, this thesis will combine the marker-trait analysis of putative neutral markers with important ornamental traits of cultivated chrysanthemum with the analysis of candidate genes involved in the shoot branching pathway being the most relevant phenotypic trait here.

1.3 Shoot branching is shaping plant architecture

The plant architecture attracts considerable attention in the developmental biology because complete knowledge of processes in a plant holds the promising future of designing plants with an ideal phenotype (Wang and Li, 2008). The above ground habit of a plant is mainly determined by the activity of the shoot apical meristem (SAM) and meristems of axillary buds (AM). The formation and outgrowth of AMs allows a plant to adapt to environmental changes and is precisely regulated. In chrysanthemums it is directly associated with the ornamental value, as it shapes the inflorescence and the growth habit. While the regulation of shoot branching of model plants was already extensively described, there is only very limited information on shoot branching available in the chrysanthemum.

1.3.1 The control of shoot branching in plants

The control of shoot branching in plants is a complex process that is affected by (I) the initiation of axillary meristems (AM) and (II) their outgrowth. These processes are determined by the specific genetic background, the expression of genes, the activity of phytohormones and interactions with the environment.

Major progress in understanding the underlying processes were conducted in the model plant *Arabidopsis thaliana*. Therefore, it is known that the initiation of shoot branching starts with the establishment of the axil identity and the maintenance of the meristem formation competence. For that reason several genes, especially the transcription factor *LATERAL SUPRESSOR* (*Ls*), have to be locally accumulated and expressed (e. g., reviewed by Schmitz and Theres (2005) or Barton (2010)). Afterwards the meristem starts to organise with the downregulation of *Ls* and for example the focused expression of the *SHOOT MERSITEMLESS* (*STM*) gene and the *REVOLUTA* (*REV*) gene (Schmitz and Theres, 2005). Furthermore, the whole process is influenced by the phytohormone auxin (McSteen, 2009). The outgrowth of an AM is then thought to impose three different states of dormancy: (I) endodormancy, by factors acting directly within the bud, (II) paradormancy, by signals within the plant but external to the bud, and (III) ecodormancy, by environmental factors external to the plant (Lang *et al.*, 1987).

Recently, strigolactone (SL) was characterised as a new phytohormone, which controls the outgrowth of side shoots and thus the breaking of bud dormancy (Gomez-Roldan *et al.*, 2008, Umehara *et al.*, 2008). It was identified after exhaustive mutant screenings in *A. thaliana*, pea (*Pisum sativum*), petunia (*Petunia* × *hybrida*) and rice (*Oryza sativa* L.). Its role seems to be generally conserved in plants. These mutant screenings led to the identification and biochemical characterisation of several pathway genes, such as *CCD*7/*MAX*3, *CCD*8/*MAX*4 (both carotenoid cleavage dioxygenases), *MAX*1 (a P450 cytochrome) and *MAX*2 (F-box receptor).

SL is synthesised from carotenoids into a mobile intermediate induced by CCD7 and CCD8 (Schwartz *et al.*, 2004), which is then further modified by the cytochrome P450 (Stirnberg *et al.*, 2002). There might be yet further unknown enzymatic steps involved in the synthesis of SL.

To become biologically active, SL has to be recognised by MAX2, which is a F-box receptor of the leucine-rich repeat (LRR) protein family and functions in the ubiquitin-mediated degradation of target proteins (Stirnberg *et al.*, 2007). As it is the case with cytokinin (CK), SL is transported acropetally through the xylem (Kohlen *et al.*, 2011), but it can also be synthesized locally in axillary buds. For the regulation of bud outgrowth SL interacts with the phytohormones auxin and cytokinin. Currently, two different modes of action are hypothesised: (I) a direct and (II) an indirect one. SL and CK act directly in the buds to control their outgrowth through the joint regulation of the TCP transcription factor BRANCHED1 (BRC1) that inhibits the outgrowth (Braun *et al.*, 2012; Dun *et al.*, 2013). In contrast, bioactive SL acts indirectly via auxin. By altering the polar auxin transport (PAT) SL reduces the capacity of the PAT stream in the main stem, leading to enhanced competition between buds to release their auxin into the stem and promoting their outgrowth (Crawford *et al.*, 2010; Shinohara *et al.*, 2013). Moreover, auxin is transported basipetally and regulates the biosynthesis of SL in a feedback loop (Hayward *et al.*, 2009). However, the whole process of the regulation of bud outgrowth is not fully understood yet.

1.3.2 Shoot branching in chrysanthemum

Shoot branching is responsible for the architecture of the plant and thus mainly for its aesthetic value. Decorative chrysanthemum cultivars are characterised by one flower per stem. Therefore, axillary flower buds have to be removed manually during cultivation, which is laborious, time consuming and expensive. Consequently, the breeding of cultivars expressing a non-branching trait (see Figure 1.2) would reduce manual labour requirements and subsequently the costs of cut flower production. As a result, shoot branching is already investigated from different points of view in chrysanthemums, such as environmental factors, the identification of candidate genes, or the regulation on the phytohormonal level. The major findings of the according studies are briefly reviewed as follows.

Environmental factors affecting shoot branching in chrysanthemum

Matsumoto (1994a) reported that non-branching in chrysanthemum can be induced by hot temperature (more than 30 °C in the daytime/25 °C at night). However, this characteristic was not stable and low temperatures restored the branching. Thereby, the time point of the harvest of cuttings and the cultivation of mother plants resulted in varying branching traits in the same cultivar (Matsumoto, 1994b).

Since chrysanthemums form inflorescences consisting of several flowering single stems, branching during the vegetative phase is different from branching during the generative phase. From previous studies it is known that the number of flowers and branching increases with assimilate availability (Carvalho and Heuvelink, 2003), increasing temperature (Carvalho *et al.*, 2005) and with increasing light intensity (Kang *et al.*, 2012). The density of plantation influences branching as well. Higher densities lead to a stronger competition of neighbouring plants for light resulting in a focused growth of the primary stem.

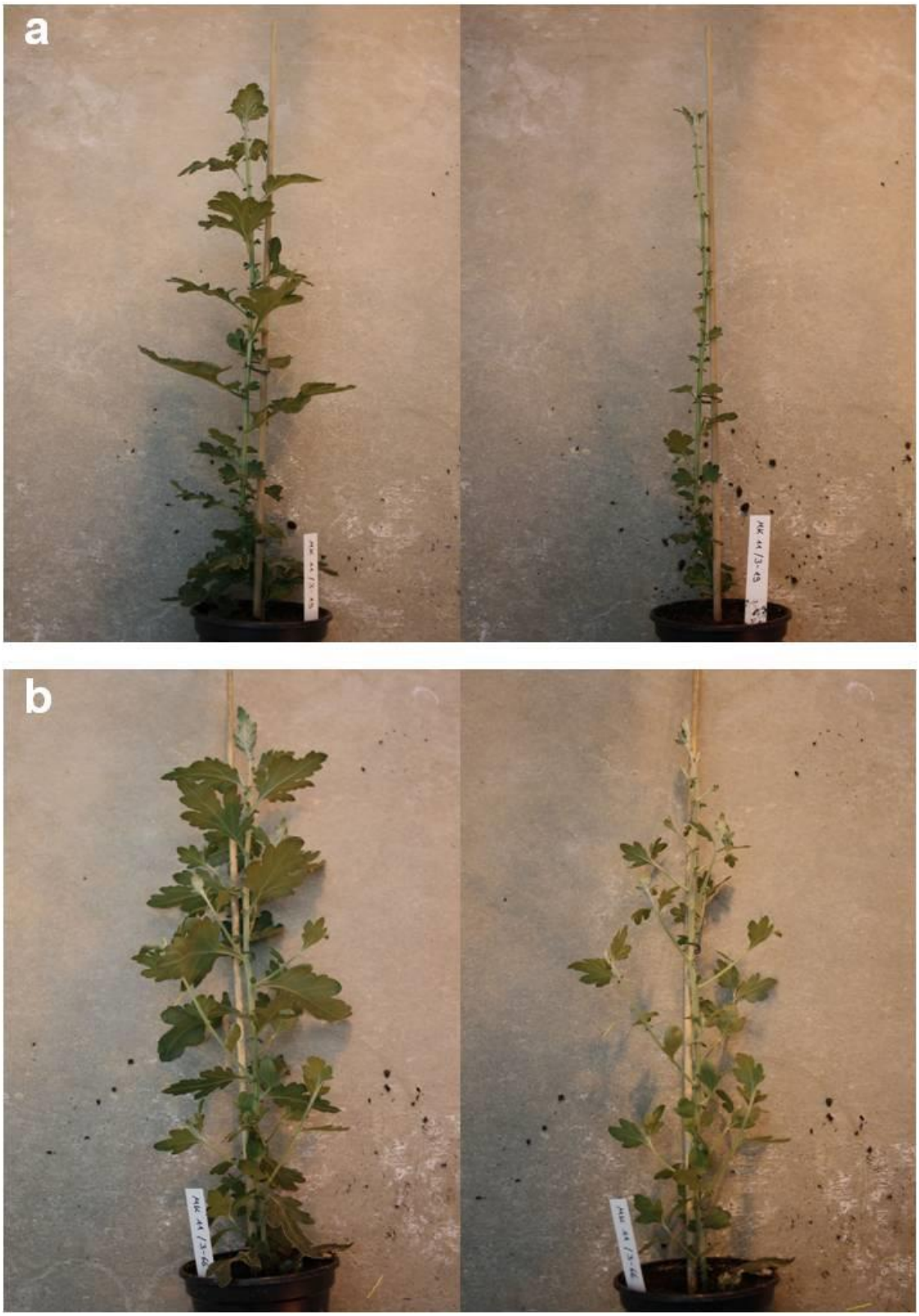

Figure 1.2: Shoot branching phenotypes of chrysanthemum: a) weak branched phenotype and b) strong branched phenotype. Plants where photographed during the vegetative phase and are of the same age. While the left picture represents the intact plants, leaves where removed from these plants in the right picture.

Identification of candidate genes associated with shoot branching in chrysanthemum

In addition to environmental factors the genotype of a plant influences shoot branching. For instance, some varieties of chrysanthemum that are used as cut flowers, show less branching than others (Tetiedt, personal communication).

Loci for candidate genes that affect branching in chrysanthemum, have already been specified by the identification of several functional genes for the initiation and outgrowth of AMs. Yang *et al.* (2005) characterised the *LATERAL SUPRESSOR-LIKE* (*DgLsl*) gene from chrysanthemum. This information was used to generate non-branching chrysanthemum lines by a transgenic approach (Han *et al.*, 2007). These plants that were transformed with an antisense construct of the *DgLsl* gene, showed less branching because of their reduced capacity to initiate AMs (Han *et al.*, 2007). A following study by Jiang *et al.* (2010) demonstrated that the suppressed expression of *DgLsl* modulated the auxin and gibberellic acid content. The content of both phytohormones was enhanced, so that *DgLsl* seems to control shoot branching through its effect on auxin and gibberellic acid levels (Jiang *et al.*, 2010).

Recently, several genes of the SL pathway were identified in chrysanthemums. Liang *et al.* (2010) characterised the *CCD*8/*MAX*4 gene of chrysanthemum. The chrysanthemum *CCD*8 orthologue complemented the *A. thaliana MAX*4-1 mutant phenotype and the expression of *CCD*8 was up-regulated by the application of exogenous auxin and down-regulated by exogenous SL in chrysanthemum (Liang *et al.*, 2010). Additionally, three orthologues of *MAX*2 that encodes the F-Box receptor for SL signalling, were identified. The function of one orthologue was confirmed by restoring the phenotype of the *MAX*2-1 mutant of *A. thaliana* (Dong *et al.*, 2013). Other SL pathway genes, such as *CCD*7/*MAX*3 or *P*450/*MAX*1, have not yet been characterised and might be interesting targets to improve our knowledge about shoot branching in chrysanthemum.

Not only have the aforementioned genes that directly affect the SL pathway already been characterized, but also the *BRC*1 gene acting downstream of the SL pathway (Chen *et al.*, 2013). Its function was confirmed by complementing an *A. thaliana* mutant phenotype; its expression was altered by the planting density, the PAT and the exogenous application of auxin (Chen *et al.*, 2013).

In addition to, the effects on shoot branching of the expression of two foreign genes in the chrysanthemum genome have been examined (Aswath *et al.*, 2004; Khodakovskaya *et al.*, 2009). The introduction and over-expression of a *MADS*4 transcription factor of sweet potato (*Ipomoea batatas*) increased axillary shoot formation in chrysanthemum (Aswath *et al.*, 2004). Another construct using an *isopentyl transferase* (*ipt*) gene of *A. thaliana* under the control of the *LEACO*1 gene promotor from tomato (*Solanum lycopersicum*) increased the flower number in chrysanthemum after transformation (Khodakovskaya *et al.*, 2009). The *ipt* gene is involved in the biosynthesis of CK and the transgenic chrysanthemum lines accumulated higher concentrations of CKs (Khodakovskaya *et al.*, 2009). Therefore, the change in the CK level might have promoted the increase in flowering side shoots.

Currently, nearly all investigative approaches for shoot branching in chrysanthemum aim to generate transgenic plants with altered expression of functional genes leading to an increase or decrease in shoot branching. Those plants might be valuable tools to improve the breeding process in chrysanthemum, but the introduction of transgenic plants might be costly due to regulatory processes and debatable on the consumer side. Therefore, the question to which extent DNA polymorphisms of candidate genes describe the phenotypic variation in shoot branching in chrysanthemum populations should be answered.

Phytohormones and shoot branching in chrysanthemum

As mentioned above, candidate genes have been characterised in chrysanthemum, which are involved in the synthesis (e. g., *CCD*8 or *ipt*) or the regulation (e. g., *DgLsl*) of phytohormones. This highlights the fundamental role of phytohormones, such as SL, auxin and CK, and their crosstalk in the control of shoot branching.

A study of Jiang *et al.* (2012) documented changes of the endogenous hormones auxin and CK in lateral buds during their outgrowth. Different ratios between auxin and CK were detected in two varying cultivars. The cultivar with strong apical dominance (the SAM suppresses the emergence of AMs) and less-branching had a higher auxin content than the cultivar with weak apical dominance. Instead the latter cultivar was characterised by a higher CK content (Jiang *et al.*, 2012).

Furthermore, the auxin level of both cultivars decreased remarkably with flower induction. Hereby, the apical dominance collapsed and nearly all existing axillary buds grew out (Jiang *et al.*, 2012).

In contrast, exogenous application of synthetic SL reduced the bud outgrowth in chrysanthemum (Liang *et al.*, 2010). However, in the presence of an auxin source SL suppressed more effectively the outgrowth of axillary buds (Liang *et al.*, 2010). Thus, the chemical treatment of plants with synthetic SL analogues, such as GR24 (Johnson *et al.*, 1981) or CISA-1 (Rasmussen *et al.*, 2013), might be useful to control shoot branching during the cultivation of chrysanthemums in the future.

1.4 Thesis objectives

Genetic and genomic investigations in chrysanthemum are difficult due to its large and complex genome structure. The analysis of molecular markers allows a glimpse into the genome and reveals mechanisms of inheritance of an organism. Thereby, this thesis aims to investigate the genetics of important morphological traits in chrysanthemum using molecular markers. Especially, shoot branching will be thoroughly considered by focusing on the role of the SL pathway. As a prerequisite for these analyses, molecular marker data are generated and used not only to investigate the inheritance of loci, but also to reveal the genetic structure of a diverse association panel. Hence, this thesis will focus on the following major objectives:

i) to determine whether marker loci are inherited in a disomic manner, as it would be expected for an allopolyploid taxum

ii) to analyse the relatedness of chrysanthemum varieties and selected species on the basis of multilocus AFLP® markers in order to calculate genetic similarity indices

iii) to monitor the characteristic of important ornamental traits, especially focusing on shoot branching, in a collection of diverse chrysanthemum varieties and a biparental F1 population

iv) to identify marker-trait associations with important ornamental traits

v) to understand, to which extent DNA polymorphisms of candidate genes with known function in the regulation of bud outgrowth describe the phenotypic variation in shoot branching in different chrysanthemum populations

vi) to characterise the *CCD7* gene of chrysanthemum hybrids

vii) to investigate the role of the SL pathway in shoot branching on a molecular level

The thesis comprises of four chapters and a general discussion including conclusions and outlook. The individual chapters are connected to the main objectives (the first chapter deals with the first objective, the second chapter with objective number two, the third chapter with third, fourth and fifth objectives and the last chapter with the remaining ones). Progress break down of the thesis (Figure 1.3) depicts that the analysis of the type of ploidy and the genetic diversity within chrysanthemum hybrids are prerequisites for genetic analyses of important ornamental traits.

The individual chapters are planned for subsequent publication or have already been published in peer-reviewed journals.

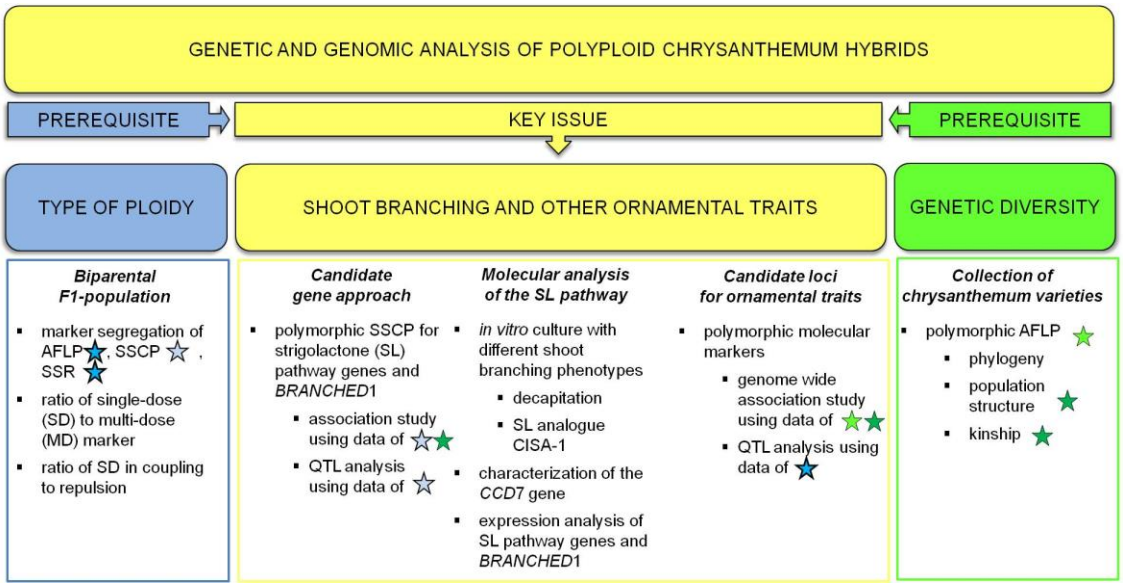

Figure 1.3: Workflow for the genetic and genomic analysis of polyploid chrysanthemum hybrids with emphasis on shoot branching. The figure summarizes the main objectives of this thesis and indicates the methods used to achieve them. The analyses of the type of ploidy and the genetic diversity in a collection of chrysanthemum varieties are prerequisites for the genetic analysis of shoot branching and other ornamental traits. Abbreviations: amplified fragment length polymorphism (AFLP®), single-strand conformation polymorphism (SSCP), microsatellite (SSR), quantitative trait locus (QTL)

2 The type of ploidy of chrysanthemum is not black or white: a comparison of a molecular approach to published cytological methods

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Abstract

Polyploidy is a widespread phenomenon among higher plants and a major factor shaping the structure and evolution of plant genomes. The important ornamental chrysanthemum (*Chrysanthemum indicum* hybrid) possesses a hexaploid genome with 54 chromosomes and was classified based on its evolutionary origin and cytological methods as an allopolyploid. However, it is questionable whether cytological methods are sufficient to determine the type of ploidy, and there are more informative methods available based on molecular marker analyses. Therefore, we collected segregation data for 406 dominant molecular marker alleles (327 amplified fragment length polymorphism [AFLPs], 65 single-strand conformation polymorphism [SSCPs] and 14 microsatellites [EST-SSRs]) in a biparental F1 population of 160 individuals. We analysed these data for the characteristics that differ between allopolyploids and autopolyploids, including the segregation ratio of each marker, the ratio of single-dose (SD) to multi-dose (MD) markers, the ratio of SD markers in coupling to those in repulsion and the banding patterns of the SSRs. Whereas the analysis of the segregation ratio of each polymorphic marker indicated disomic (13 markers) as well as hexasomic (eight markers) inheritance, the ratio of SD markers in coupling to those in repulsion was 1:0, which is characteristic of autopolyploids.The observed ratio of SD to MD markers was close to 0.7:0.3which is significant different to the assumed segregation for auto- and allohexaploids. Furthermore, the three EST-SSR alleles were inherited in all possible combinations and were not independent of each other, as expected for fixed heterozygosity in

allopolyploids. Combining our results with published cytological data indicates that cultivated chrysanthemums should be classified as segmental allo-hexaploids.

Key words

allopolyploidy, autopolyploidy, molecular marker, polysomic inheritance

Introduction

Chrysanthemums (*Chrysanthemum indicum* hybrid, *C.* x *grandiflorum* or *C. morifolium*) are among the most economically important ornamental plants worldwide and are produced as cut flowers and as potted or garden plants. Chrysanthemums belong to the large plant family *Asteraceae* and are native to the Northern Hemisphere, primarily Europe and Asia (Dowrick, 1952). Cultivated chrysanthemums are generally believed to be the result of natural hybridisation involving several different species, such as *C. indicum* L., *C. morifolium*, *C. vestitum* and *C. lavandulifolium* (Vogelmann, 1969; Dai et al., 1998; Yang et al., 2006). These crosses led to the formation of a hexaploid hybrid complex with 54 chromosomes (Dowrick, 1953).

Because cultivated chrysanthemums resulted from hybridisation events between different species, and because the occurrence of bivalent chromosomes is detected in meiosis in all four investigated polyploid chrysanthemum accessions (Watanabe, 1977; Li et al., 2011), the cultivated forms are currently classified as allo-hexaploids. However, polyploid genomes can be highly dynamic, and Stebbins (1947) proposed that it might be difficult to unambiguously classify the type of ploidy of an organism. This was also indicated by Watanabe (1983) for the hexaploid *Chrysanthemum japonense*, which is not believed to be a progenitor of the *C. indicum* hybrid, reporting a very limited formation of multivalents (3.8%) using microscopic methods. In contrast, Watanabe (1977) and Li et al. (2011) state a clear autopolyploid behavior in cytological studies of *Chrysanthemum* species closely related to the ornamental types. Therefore, it is necessary to combine cytological and molecular methods to clarify the type of ploidy.

Polyploids are classified into the two major categories of auto- and allopolyploids. Allopolyploids are characterized by preferential pairing of chromosomes or fixed heterozygosity, which results from the combination of divergent parental genomes, bivalent chromosome formation in meiosis and disomic inheritance at each locus. In contrast, for autopolyploids the formation of multivalent chromosomes and polysomic inheritance is generally assumed (Stebbins, 1947; Soltis and Soltis, 2000). However, in addition to these extremes, intermediary forms have also been described (Stebbins, 1947; Sybenga, 1969).

In addition to cytological methods, Wu et al. (1992) described the usefulness of single-dose (SD) molecular markers to distinguish allopolyploidy from autopolyploidy. SD markers are characterized by only one dominant marker allele at a single locus and can be distinguished from multi-dose markers (MD) by determining the means of the corresponding recombination frequencies (Mather, 1957). Da Silva et al. (1993) determined the theoretical ratios of SD to MD markers for allo- and autopolyploids, which might indicate the ploidy type of an organism. A single dose marker present in only one parent (uniparental marker) has a theoretical segregation ratio of 1:1 (presence: absence) in an F1 progeny of both autopolyploids and allopolyploids. Likewise, biparental markers will segregate in a 3:1 (presence:absence) ratio in both auto- and allopolyploids. In contrast, multidose markers have more complex segregation ratios that differ between autopolyploids and allopolyploids. The expected ratios for singledose to multidose markers is 0.56:0.44 in allopolyploids and 0.7:0.3 in autopolyploids (Da Silva et al. 1992) so that the type of ploidy can be inferred if a larger number of markers is tested for singledose versus multidose segregation.

Furthermore, Wu et al. (1992) used SD markers for 75 individuals and showed a linkage in the coupling phase for allo- and autopolyploids, whereas a linkage in the repulsion phase can be detected only in allopolyploids. By calculating the ratio of markers in coupling to those in repulsion, it is possible to distinguish allopolyploidy (ratio of 1:1) from autopolyploidy (ratio of 1:0 for polyploids above tetraploidy). Additionally, the banding patterns of

sequence specific markers, such as SSRs, reflect the distributions of the homologous and homeologous chromosomes within the progeny. Thus, this type of marker is informative in determining the pairing of the chromosomes, as it indicates the occurrence of fixed heterozygosity and therefore also the type of ploidy.

By using molecular markers, we sought to determine whether the classification of chrysanthemum as allo-hexaploid, based on cytological methods, is conclusive. Knowledge about the type of ploidy is of interest from an exploratory and a breeder's point of view because desirable alleles cannot be freely combined in allo-hexaploid genotypes. Therefore, we describe the use of amplified fragment length (AFLP), single-strand conformation polymorphism (SSCP) and microsatellite (SSR) markers in a segregating biparental F1 population to investigate the type of ploidy of cultivated chrysanthemums. Additionally, we compare our results with previously published cytological data.

Material and Methods

Plant material

We established a segregating biparental F1 population (MK11/3) of 160 individuals by crossing the female parent *C. indicum* hybrid 'Kitam' (541) with the paternal parent 'Relinda' (VZR), which is a registered *C. indicum* hybrid variety. One cutting of each genotype was cultivated in each of three independent randomised blocks with 48 plants per m^2 in plots of 12.5 cm × 12.5 cm. The plants were grown in a fertilised substrate (a mixture of peat moss and chalked compost soil) in a greenhouse under a 16 h light/8 h dark cycle at a constant temperature of 22°C.

DNA extraction

For the DNA extraction, 70 mg of unfolded, young leaves was used. The plant material was dried overnight at 37 °C, frozen in liquid nitrogen and ground using a bead mill. The extraction was performed using the NucleoSpin Plant II Kit from Macherey and Nagel (Düren, D) following the manufacturer's instructions, with minor modifications. The concentration of genomic DNA

was assessed spectrophotometrically at 260 nm and was evaluated for purity by determining the OD 260 nm/280 nm and the OD 260 nm/230 nm ratios. The DNA quality was assessed by agarose gel electrophoresis.

Marker analysis

AFLP® analysis

The AFLP® analysis was performed as described previously (Vos *et al.*, 1995), with minor modifications according to Klie *et al.* (2013). For each sample, 100 ng of DNA was digested with 9 U *Hind*III (Fisher Scientific - Germany GmbH, Schwerte, D) and 3.5 U *Mse*I (Fisher Scientific - Germany GmbH, Schwerte, D). The preamplification reactions were performed with specific primers that had an A as a selective base at the 3' end (*Hind*III [5'-AGACTGCGTACCAGCTT-A-3'] and *Mse*I [5'-GACGATGAGTCCTGAGTAA-A-3']). *Hind*III (5'-AGACTGCGTACCAGCTT-ANN-3') primers with two extra selective bases and *Mse*I (5'-GACGATGAGTCCTGAGTAA-ANNN-3') primers with three extra selective bases were used for the final amplification. The *Hind*III primers were end-labelled with an infrared dye (either IRD 700 or IRD 800; Eurofins MWG, Ebersberg, D). In a single PCR reaction, labelled primers were used either as single primers or in combinations of two differently labelled primers (IRD 700 and IRD 800). In total, 21 selective primer combinations were analysed (Table 2.1). The fragments were separated on 6 % polyacrylamide gels (Sequagel XR, Hessle, UK) using a DNA Analyzer (LI-COR, Lincoln, NE, USA) and automatically processed using the e-Seq-Software (V3.0, LI-COR, Lincoln, NE, USA).

SSCP markers for candidate genes

Mutant screens in *Arabidopsis* and other plants identified several genes that control shoot branching and are involved in strigolactone biosynthesis and perception. Some of these genes, such as *CCD*8 (Liang *et al.*, 2010), *MAX*2 (Dong *et al.*, 2013) and *BRC*1 (Chen *et al.*, 2013), have also been characterised in *Chrysanthemum*. In addition, we isolated a *CCD*7 homologue from *Chrysanthemum* (unpublished) and screened this sequence and those of the other genes containing polymorphisms using single-strand conformation polymorphism (SSCP) analysis. Several primer pairs were used that covered

Table 2.1: The primer combinations used for the AFLP® analysis. Only the selective bases are listed in the table below. The framework of the selective HindIII primers was 5'-AGACTGCGTACCAGCTT-NNN-3', and that of the selective MseI primers was 5'-GACGATGAGTCCTGAGTAA-NNNN-3'.

***Hind*III- IRD 700**	***Hind*III- IRD 800**	***Mse*I**
AGC	AGT	ACCG
AAT	AGT	ACAG
AAT	ACG	ATGG
AGC	ACA	ACAT
AAT	-	ACGA
AGA	-	ACGG
AGT	-	ATAG
AAC	-	ACCT
AAT	-	ATGA
-	ACA	AAGC
-	ACG	AGCA
-	ACG	AAGC
-	ACG	ACGA
-	ACG	ACAC
-	ACG	ATCA
	ACA	ACCA
	ACA	ACAG

various fragments of each candidate gene (see Table 2.2). Most of the PCR products were IRD-labelled using the universal M13 sequences (5'-GTAAAACGACGGCCAGT-3' for the forward primer and 5'-CAGGAAACAGCTATGAC-3' for the reverse primer) at the 5' end (Schuelke, 2000). The PCR conditions were as follows: 0.2 µM of each unlabelled primer, 0.07 µM of each labelled primer and also a M13 primer end-labelled with either the IRD 700 dye or the IRD 800 dye (Eurofins MWG, Ebersberg, D) in a final 25 µL reaction volume (2x Williams Buffer, 0.16 mM dNTPs, 0.7 U DCS-Taq polymerase [Enzymatics, Beverly, Massachusetts, USA] and 30 ng template DNA). The conditions of the PCR amplification were as follows: 95 °C (3 min), then 25 cycles at 94 °C (30 s) / 58 °C (30 s) / 72 °C (45 s),

followed by eight cycles at 94 °C (30 s) / 52 °C (45 s) / 72 °C (60 s), and a final extension at 72 °C for 10 min. All other PCR products were amplified by a standard PCR reaction in a final reaction volume of 20 µL containing 1x Williams Buffer, 0.2 mM dNTPs, 0.5 µM primers, 0.5 U DCS Taq polymerase and 30 ng template DNA. The conditions of the PCR amplification were as follows: 95°C (3 min), then 30 cycles at 94 °C (30 s) / 60 °C (60 s) / 72 °C (60 s), followed by a final extension at 72 °C for 10 min. An equal volume of SSCP dye (95 % formamide, 0.01 M NaOH, 0.05 % xylene cyanol and 0.05 % bromophenol blue) was added to each PCR reaction, and this step was followed by denaturing the samples for three min at 95 °C. The denatured samples were immediately placed on ice prior to loading onto cooled (10 °C) 0.5 x MDE gels (0.5x MDE® gel solution [Lonza Group Ltd., Basel, SUI], 0.6x long run TBE [80.4 mM Tris, 7.5 mM Borsäure, and 1.5 mM EDTA], 8.3 % glycerine, 0.05 % APS, 10 µL TEMED and *ad* 15 mL water). IRD-labelled single strands were detected with the Odyssey® Infrared Imaging System (LI-COR, Lincoln, Nebraska, USA) and automatically documented using Odyssey Software (V3.0, LI-COR, Lincoln, Nebraska, USA). The non-IRD-labelled single strands were visualised by silver staining according to the protocol of Sanguinetti *et al.* (1994).

EST-SSR markers

Sequence information for 7009 ESTs from *Chrysanthemum morifolium* was downloaded from NCBI (November 2010). These ESTs were screened for mono-, di-, tri-, tetra-, penta-, hexa- and hepta-nucleotide motifs of microsatellites with a copy number of at least four repeats using the TandemRepeatFinder (Benson, 1999). For the 21 SSR-containing ESTs, primer pairs were designed using the Primer3Plus software (Untergasser *et al.*, 2007) with the default settings. Each forward primer was extended by a universal M13 sequence tag (5‘-GTAAAACGACGGCCAGT-3') at the 5' end for IRD-labelling of the PCR fragments (Schuelke, 2000). The three EST-SSR markers (

Table 2.3) were used on the entire population using the PCR conditions as described previously. The PCR products were separated on 6 % polyacrylamide gels (Sequagel XR, Hessle, UK) using a DNA Analyzer (LI-COR, Lincoln, Nebraska, USA) and automatically documented using e-Seq-Software (V3.0, LI-COR, Lincoln, Nebraska, USA).

Data analysis

The marker banding patterns for each genotype were visually scored as present (1), absent (0) or ambiguous (?).

According to Mather (1957), the uniparental and biparental markers were classified as single-dose (SD) or multi-dose (MD) markers using the geometric means between the two segregation distributions. For the uniparental markers, the geometric mean was calculated between the 1:1 and the 3:1 distribution by the formula $\sqrt{\frac{3}{1}\times\frac{1}{1}}=1.73$ as the point for selection, whereas for the biparental markers, the mean between the 3:1 and 15:1 distribution was determined by the equation $\sqrt{\frac{15}{1}\times\frac{3}{1}}=6.71$ for selection (Grivet *et al.*, 1996). For each marker, the recombination frequency was calculated and compared to the corresponding selection point. Markers with ratios below this point were classified as SD markers, and those with ratios above the threshold were classified as MD markers. Da Silva et al. (1993) estimated the theoretical proportion of SD to MD markers as 0.56 to 0.44 for allopolyploidy and 0.7 to 0.3 for autopolyploidy. We compared our ratios to these ratios using the chi-square test in R software (version 2.15.2; R Core Team 2012).

Table 2.2: A list of the primer pairs for the candidate genes *CCD*7, *CCD*8, *MAX*2 and *BRC*1 used in the single-strand conformation polymorphism (SSCP) analysis. The gene, GB accession (if available), primer sequence (5' to 3'), size of expected PCR product and detection method are given. Primers marked by an asterisk contained a universal M13 sequence (5'-GTAAAACGACGGCCAGT-3' for forward primers and 5'-CAGGAAACAGCTATGAC-3' for reverse primers) at the 5' end for infrared (IRD) labelling of the PCR fragments. Those fragments were detected via infrared imaging, whereas non-labelled fragments were detected via silver staining.

Gene	**Accession**	**Primer Pairs**		**Product Size**	**Detection Method**
CCD7	unpublished	F	CCCTCTAGATGGTCATGG	550 bp	silver staining
		R	AGCAAGATCTAACAAGTCCACACCAC		
		F*	TGTCATGCAACGCAGAGGAT	1750 bp	M13-IRD700
		R	CCCACATTTGAGAAGGAGCTT		
		F	GGTGGGGCCCCTTACGAGAT	600 bp	silver staining
		R	GCATTGCATGACATCATAAG		
		F*	TCCATGACTGGGCTTTCACA	380 bp	M13-IRD700
		R	CCCACATTTGAGAAGGAGCTT		
CCD8	Liang *et al.*, 2010	F*	ATGGCATCCTGAGTCGAAAG	550 bp	M13-IRD700
		R	GCGTCTACTAGTTCTCCCTTTGG		
		F*	ACAAGCTGCGGCTTCAAA	260 bp	M13-IRD700
		R	GCGTCTACTAGTTCTCCCTTTGG		
		F*	GGTGCGTCCCTAACTGACAA	480 bp	M13-IRD700
		R	GACTCAGGATGCCATTCAAAC		
MAX2	JX556222	F*	GCCAATCCAGGGTCGGATAC	550 bp	M13-IRD700
		R	GTAACGACAAACTCCTCTGG		
		F*	ATGTCTTTCTCCACCACAACAAT	1400 bp	M13-IRD700
		R	AAGCCTACTCGCACTCAACG		
BRC1	JX870411	F	TGCAGCATCAGTTCAGTGACT	380 bp	M13-IRD700
		R*	AGCAGTAGCATACAATTGACATAGT		

Table 2.3: A list of three EST-SSR markers used on the chrysanthemum MK11/3 population. The GB accession of the chrysanthemum EST, primer sequence (5' to 3'), size of the expected PCR product, motif and number of repeats are given.

Accession	Forward Primer	Reverse Primer	Product Size	Motif	Copy Number
69838459	CCTCTCCTCCCAACAAACAA	CCGTAAGTGCCTTCACCAAT	209 bp	AAG	8
69834897	CCGCTACAATTCAAACAAACAA	GTGGTGGTGGTTGAGAACCT	207 bp	AATCCA	5
69837400	CCAATTGAGGCGTTTTGTTT	CATTTTCCACGTAAGCACCA	239 bp	GGT	10

By determining the ratio of SD markers in coupling to those in repulsion in a population of 75 individuals, Wu *et al*. (1992) distinguished allopolyploidy from autopolyploidy. We estimated this ratio using the previously selected uniparental SD markers of the MK11/3 population for each parent. We generated linkage maps with a maximal recombination frequency of 0.35 for 75 randomly selected offspring in the backcross-1 (BC1) mode of JoinMap version 4 (van Oijen, 2006). The markers were placed into linkage groups based on their independent LOD values, which ranged from 4 to 10. The marker distances in centimorgan were calculated using Kosambi's mapping function. Subsequently, the values of the marker data matrix were inverted so that the present bands were coded as absent and the absent bands were coded as present. These inverted markers were integrated into the previously calculated maps. The markers that were linked in the original dataset were designated to be in coupling, and the markers that showed linkage between the original and the inverted datasets were designated to be linked in repulsion (Ukoskit and Thompson, 1997; Kriegner *et al.*, 2003). The resulting ratio of markers in the coupling to the repulsion phase was compared to the assumed ratios (Wu *et al.*, 1992) for allopolyploidy (1:1) and autopolyploidy (1:0 for polyploids above tetraploidy) using the chi-square test in R software (version 2.15.2; R Core Team, 2012).

Results

Molecular marker data for the MK11/3 population

Allo- and autohexaploids differ in their segregation ratios, their ratios of marker dosage and their ratios for markers in coupling to those in repulsion. Therefore, we used various molecular markers, such as AFLP®, SSCP and SSR markers, to investigate the inheritance patterns in chrysanthemum.

All of the segregating marker fragments were analysed dominantly because of the complex banding patterns for even single-locus markers, such as SSR or SSCP markers, in a hexaploid genome. In total, 406 polymorphic markers were scored in the MK11/3 population. The vast majority were AFLP markers with 326 fragments derived from 21 primer combinations, followed by 73 SSCP marker fragments for the candidate genes *CCD*7 (29 fragments), *CCD*8 (16 fragments), *MAX*2 (eight fragments) and *BRC*1 (12 fragments) and 14 DNA fragments derived from the three EST-SSRs.

Marker segregation types of 1:0 or 7:1 are expected for an allopolyploid organism, and types of 4:1 or 9:1 are expected for an autopolyploid organism. Accordingly, all of the polymorphic markers were tested to determine whether their segregation ratios were consistent with autopolyploidy or allopolyploidy by the chi-square test (Table 2.1). For 204 of the total markers significant possible segregation types were assigned by the statistical test. Not all markers could be assigned because a large number of individuals is needed to clearly distinguish between different segregation types. The 1:1 segregation pattern does not distinguish between the types of ploidy and is therefore not informative. A large proportion of the markers (34) displayed a skewed segregation and did not fit to any of the ratios diagnostic for allo- or autopolyploidy. In total, 13 markers segregated in a disomic manner, whereas eight markers showed a 4:1 ratio that is characteristic of hexasomic inheritance. Therefore, there are more markers indicating a disomic inheritance, as expected for an allo-hexapolyploid genome.

Table 2.4: The segregation ratios for the MK11/3 population. Only markers that were assigned a segregation ratio expected for uniparental and biparental markers by the chi-square test (1-α = 0.95; df = 1) are shown.

Parental composition	Segregation ratio	Number of markers	Type of segregation
Maternal	1:1	63	Non-informative
Paternal	1:1	85	Non-informative
Maternal	1:2	17	Skewed
Paternal	1:2	18	Skewed
Maternal	3:1	7	Disomic, duplex x nulliplex
Paternal	3:1	5	Disomic, duplex x nulliplex
Maternal	4:1	3	Hexasomic, duplex x nulliplex
Paternal	4:1	5	Hexasomic, duplex x nulliplex
Biparental	7:1	1	Disomic, duplex x simplex

The segregation patterns of SSR marker fragments

The three SSR markers amplified four (marker 69838459) or five (markers 69834897 and 69837400) fragments. An example of the segregation pattern of the EST-SSR marker 69834897 is given in Figure 2.1. This marker amplified four alleles (a, b [double band] and c), which were polymorphic between the maternal (541) and paternal parents (VZR). For all three EST-SSRs, an independent assortment of the amplified alleles was observed, as expected for polysomic inheritance. No cosegregation of specific alleles was observed, nor was any allele combination found to exclude another, as would be expected in the case of disomic inheritance.

Marker dosage ratios

Of the 406 segregating uni- and biparental markers, 273 were classified as SD markers and 133 were classified as MD markers according to their segregation ratios (Mather, 1957). The ratio of SD to MD markers was estimated to be 0.67 to 0.33 and was compared to the theoretical proportion

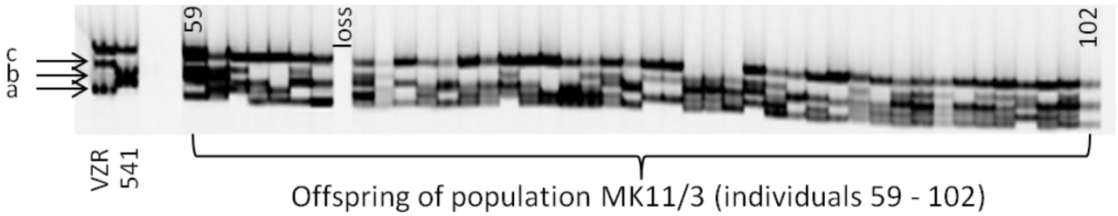

Figure 2.1: The segregation pattern of EST-SSR 69834897 for selected individuals from the MK11/3 population. Different genotypes are represented by individual lanes. The maternal parent 541 and the paternal parent VZR are shown on the left side of the gel. Polymorphic alleles between the parents are shown as a, b (double band) and c.

of SD to MD markers indicative of auto- and allopolyploidy (da Silva et al., 1993 and 1996; Ukoskit and Thompson, 1997) using the chi-square test (Table 2.5). The ratio was significantly different from the expected ratio for allopolyploidy and for autopolyploidy, also the ratio was closer to the values expected for autohexaploids.

Table 2.5: The ratios of single-dose (SD) to multi-dose (MD) markers in the MK11/3 population. The segregation ratios were compared with the theoretical proportions of SD to MD markers for allo- (0.56:0.44) and autopolyploidy (0.7:0.3) using the chi-square test (da Silva et al., 1993). Significance is indicated with *, the critical value is $\chi 2$ 0.95 (df = 1) = 3.84, and the p-values are given.

	Observed	**Expected**	
		Allopolyploid	**Autopolyploid**
Single-dose	273	253.75	284.2
Multi-dose	133	152.25	121.8
Markers in total	406	406	406
SD: MD	0.67:0.33	0.625:0.375	0.75:0.25
$\chi^2_{0.95\ (df=1)}$ **= 3.84**		1.799	0.595
p-value (α = 0.05)		0.1797	0.4405

An analysis of marker linkage

Of the previously selected 245 SD markers, 80 markers were biparental and 165 were uniparental. These uniparental SD markers (81 for the maternal parent 541 and 84 for the paternal parent VZR) we used to identify markers in the coupling and the repulsion phases by a mapping approach. For 71 (32 for 541 and 39 for VZR) of the 165 markers, we showed linkage in coupling, whereas no markers were linked in repulsion and had LOD scores greater than 1.0. Therefore, the ratio of markers in coupling to those in repulsion was 1:0 (see Table 2.6), as expected for an autopolyploid organism with a ploidy degree above tetraploidy.

Table 2.6: The ratios of the uniparental SD markers linked in coupling to those in repulsion for the MK11/3 population. The obtained segregation ratio of 71 markers in coupling to 0 markers in repulsion was compared with the theoretical proportions for auto- (1:1) and allopolyploidy (1:0) using the chi-square test (Wu *et al.*, 1992). Significance is indicated by *, the critical value was $\chi^2_{0.95\ (df=1)} = 3.84$, and the p-values are given.

	Allopolyploid		**Autopolyploid**	
	Coupling	**Repulsion**	**Coupling**	**Repulsion**
Observed	71	0	71	0
Expected	35.5	35.5	71	0
$\chi^2_{0.95\ (df=1)} = 3.84$		43.6708*		0
p-value (α = 0.05)		$3.89e^{-11}$*		1

Discussion

Based on their evolutionary origin and published cytological analyses, cultivated chrysanthemums have been mainly classified as allopolyploid plants (Watanabe, 1977 and 1983; Li et al., 2011). However, several studies raised questions regarding whether the behavior of meiotic chromosomes is an appropriate indicator of the type of ploidy and therefore if the formation of bivalents or multivalents is a reliable indicator of whether a species is genetically an autopolyploid with tetrasomic inheritance or an allopolyploid with disomic segregation. (Soltis and Rieseberg, 1986; Krebs and Hancock, 1989; Sybenga, 1996; Qu et al. 1998). With the advent of molecular markers as an informative genomic tool, Wu et al. (1992) and da Silva et al. (1993)

described effective methods based on SD markers to distinguish allopolyploids from autopolyploids. Therefore, we used molecular markers (AFLP, SSCP, and SSR,) to investigate the type of ploidy of cultivated chrysanthemums.

In total, we scored 406 polymorphic markers in the F1 MK11/3 population. Characteristic segregation ratios for allo- (e.g., 1:0 or 7:1) and autopolyploids (e.g., 4:1 or 9:1) have been established based on the type of ploidy of a genome. By using the chi-square test, the ratios of all of the segregating markers were compared to the theoretically expected segregation ratios. The vast majority of the markers (148) were not informative because they segregated in a ratio of 1:1, which is expected for a uniparental SD marker for allo- as well as autopolyploids. Additionally, 35 markers displayed skewed segregation ratios, which is a common phenomenon in plants (Mccouch et al., 1988; Gardiner et al., 1993; Wang et al., 1998) and has been reported for chrysanthemum (Zhang et al., 2010). Of the other markers, 13 segregated in a disomic manner (uniparental 3:1, 7:1 with some of them in linkage), which would be expected for an allopolyploid, whereas eight markers displayed a 4:1 ratio, which suggests a hexasomic inheritance between a duplex and a simplex marker. Indeed, it is difficult to reliably distinguish among several similar segregation ratios, as this requires a large number of individuals. Langton (1989) also described the hexasomic inheritance of the carotenoid pigmentation in chrysanthemums, but even this study was not considered as conclusive by the author himself because of conflicting results of Jordan and Reimann-Phillip (1983) on the inheritance of anthocyanin pigmentation. Also the analysis of the marker dosages, which revealed a 0.67 to 0.33 ratio of SD to MD markers, showed significant differences to the ratios expected for both, disomic (0.625 : 0.375) and hexasomic (0.75 : 0.25) inheritance (da Silva et al., 1993 and 1996; Ukoskit and Thompson, 1997).

Therefore, we analyzed the segregation patterns of three EST-SSRs in addition to the AFLP markers. For each marker, the alleles were inherited in all possible combinations and not independent of each other, as would be expected for fixed heterozygosity. This result indicates polysomic

inheritance, as expected for autopolyploids. Therefore, it is very likely that the progenitors of cultivated chrysanthemums were phylogenetically closely related (Dai et al., 1998; Wang et al., 2002).

Furthermore, we did not detect any markers linked in repulsion in our mapping approach. This result also supports our hypothesis that chrysanthemums display polysomic inheritance. Two other published mapping approaches in chrysanthemums provide no information about the type of linkage of the mapped markers (Zhang et al., 2010; Zhang et al., 2011). By increasing the number of markers, the mapping resolution could be improved, but this does not explain the lack of markers linked in repulsion in our study.

To summarize our marker results, two methods (segregation patterns of SSRs and the ratios for markers in coupling to those in repulsion) clearly showed a polysomic inheritance in chrysanthemums, as is characteristic of an autopolyploid. Nevertheless, some markers segregated in a disomic manner and the ratio of marker dosages was close to the expected ratio for disomic inheritance, but not significant. Therefore, the inheritance in chrysanthemum seems to be mainly polysomic with a random assortment of homologues, but there are a few loci with disomic inheritance as well due to a partial preferential pairing of chromosomes. This mixed inheritance has already been detected in cytological studies in chrysanthemum that reported the predominant formation of bivalent chromosomes and the occurrence of multivalent chromosomes, though only in a small proportion (Dowrick, 1953; Chen et al., 2009; Li et al., 2011). Such intermediates have also been described in strawberries (Lerceteau-Kohler et al., 2003), rapeseed (Udall et al., 2005) and yellow cress (Stift et al., 2008). Thus, we propose to classify cultivated chrysanthemums as segmental allopolyploids according to Stebbins (1947).

This change in classification is important for the breeding progress of chrysanthemums. If chrysanthemums were strict allopolyploids, the free combination of desirable alleles would not occur. In our study, we showed that most molecular markers were inherited in a polysomic manner.

Therefore, the desirable alleles can be enriched in the gene pool independently of their subgenomic origins. Finally, the complex inheritance of ornamental traits in a segmental allo-hexaploid plant limits the effectiveness of marker-assisted selection, and phenotypic selection should be prioritized.

As Stebbins noted decades ago, it might be difficult to unambiguously determine the type of ploidy of an organism. In addition to cytological methods, molecular markers are useful tools with which to investigate the type of ploidy, and the combination of both approaches might be necessary to reveal the true type of ploidy. Based on the results of cytological studies, which report the predominant occurrence of bivalent chromosomes, a disomic inheritance was postulated for chrysanthemums. In contrast to these data, our analyses of molecular markers indicate a polysomic inheritance. Therefore, we suggest changing the classification of chrysanthemums from allopolyploid to segmental allopolyploid.

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3 Lack of structure in the gene pool of the highly polyploid ornamental chrysanthemum

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Abstract

The selection of clonally propagated chrysanthemums is mostly performed on the F1 hybrids using phenotypic characters without the use of molecular information. We applied 448 AFLP® markers to a set of 81 accessions, mainly from the European gene pool, covering the different horticultural types (cut, pot and garden varieties) and originating from the most important European chrysanthemum breeders. The average pairwise genetic similarity of 0.69 was moderate. The Neighbour-Joining clustering resulted in no grouping neither to their common origin or their horticultural type nor for similarities for important phenotypic characters. The structure of the dendrogram could not be supported by bootstrap analysis. Furthermore, the network analysis using SplitsTree, principal coordinate analysis via DARwin or analysis of the population with *structure* only differentiated two clusters. Therefore, we tested the marker saturation by plotting the mean coefficient of variation for every pairwise similarity of the bootstrap analysis against the different numbers of markers. We showed that the number of markers is sufficient for a precise estimate of the genetic similarity and that the lack of bootstrap support is not due to a low genetic diversity or a lack of marker information, but most likely resulted from the breeding history of the cultivars, involving repeated backcrosses, and the exchange of genotypes between breeders.

Keywords

genetic distance, plant breeding, AFLP®, bootstrap, dendrogram, admixture

Introduction

Chrysanthemums are among the economically most important ornamental plants worldwide and are produced as cut flowers and potted and garden plants. The success of this plant is based on the abundant diversity in flower type, colour and plant architecture (Zhang *et al.*, 2010).

This diversity coincides with the genomic complexity of cultivated chrysanthemums (*Chrysanthemum indicum* hybrid or *C. morifolium*), which are part of an allohexaploid hybrid complex (Dowrick and El-Bayoumi, 1966). Cultivated chrysanthemums are generally believed to be the result of natural hybridisations between species of *C. sinense, C. erubescens, C. ornatum, C. japonense, C. makinoi, C. chanetii*, *C. vestitum*, *C. indicum*, *C. lavandulifolium* and *C. zawadskii* (Dai *et al.*, 1998; Yang *et al.*, 2006). As an out-breeding and self-incompatible ornamental (Drewlow *et al.*, 1973), cultivated chrysanthemums are highly heterozygous. Therefore, new commercial cultivars can be established by the selection of F1 hybrids resulting from biparental crosses. Additional sources of new cultivars are spontaneously occurring or artificially induced sports. The genetic variation in the natural populations of the *C. indicum* polyploidy complex in China has been analysed previously (Yang *et al.*, 2006), and it was found that multiple hybridisation and polyploidization events occurred in the *C. indicum* complex, resulting in difficulties for the systematic classification of the genus.

However, to our knowledge the genetic diversity among chrysanthemum cultivars has not been investigated in detail to date. Over recent years, RAPD (random amplified polymorphic DNA), ISSR (inter-simple sequence repeats), and SRAP (sequence-related amplified polymorphism) markers have been used to identify sports and cultivars or to build genetic maps in chrysanthemum (Wolff *et al.*, 1995; Wolff, 1996; Huang *et al.*, 2000; Zhang *et al.*, 2011). Knowledge about the genetic diversity of the current breeding material would be helpful to avoid inbreeding depression (Anderson *et al.*,

1992) and to serve the on-going need for innovations in plant improvement and production systems of chrysanthemum.

The genetic diversity can be estimated using molecular markers due to their literally inexhaustible number in a typical plant genome. Several different marker types have been used to evaluate the diversity and to assess genetic relationships in crops (Bohn *et al.*, 1999; Garcia-Mas *et al.*, 2000; Garcia *et al.*, 2004). For minor crops, with less developed genetic and genomic resources, AFLP® (amplified fragment length polymorphism) markers are the most popular markers for the study of diversity and genetic relationships. The major advantages of AFLPs® are that the markers represent a genetic sample of the entire genome and generate a large amount of data in comparison to the sequence information from genes or noncoding regions. Therefore, AFLP® markers were also used to reveal the phylogenetic relationships of complex species groups, such as roses (Koopman *et al.*, 2008), or in cultivar groups, such as *Osteospermum* and *Dimorphotheca* (Gawenda and Debener, 2009) or *Dahlia* (Wegner and Debener, 2008). Based on these studies, AFLP® markers were used for the differentiation of the *Chrysanthemum* germplasm in the present study.

A prerequisite for any interpretation of the molecular data in studies of genetic diversity or phylogeny is to gain a measurement for the reliability of the data. Felsenstein (1985) used the bootstrap procedure to empirically estimate the sampling variance associated with phylogenetic analysis, as the distribution of the variation is unknown. Although the use of bootstrap methods was controversially discussed, it remains the key method to assess the robustness of the topologies of phylogenetic trees or dendrograms displaying genetic diversity (Soltis and Soltis, 2003). Bootstrapping allows the analysis of variance of whole data sets or within portions of data sets. Therefore, it can be used to estimate both the reliability of the topology of dendrograms and the number of molecular markers (number of polymorphic bands) required to obtain a stable distance or similarity estimate for a given group of genotypes.

Here, we analyse the genetic diversity and structure within germplasm of mainly European-cultivated chrysanthemums using AFLP® markers. The measurements of the variability were obtained by different methods, such as clustering, principal coordinate analysis (PCoA) and population structure analysis. Furthermore, we evaluated the minimum number of markers needed to represent the genetic distance between the genotypes accurately using bootstrap methods and discuss both approaches in view of the possible causes of the genetic diversity within the chrysanthemum germplasm.

Materials and Methods

Plant material

We used 81 chrysanthemum genotypes from various breeding companies, comprising 76 varieties from *Chrysanthemum indicum* hybrids and 5 accessions of four species (*Chrysanthemum coccineum, Chrysanthemum maximum, Chrysanthemum haradijanii*, and two *Chrysanthemum x hortorum*). We included these other species to get an impression about the genetic diversity found within the *C. indicum* cultivars in relation to the interspecies diversity. The varieties were 52 cut-type chrysanthemums and 9 garden chrysanthemums from 12 different breeders, 12 pot-type chrysanthemums and 3 garden mums. The plants differed in one or more traits, such as the growth rate, flower size, flower colour or branching rate (see supplemental Table S3.1). The plants were cultivated in 7.5 L pots with a fertilised substrate ("Einheitserde T") in a greenhouse under a 16 h light/8 h dark cycle at a constant temperature of 22 °C.

DNA extraction

For the DNA extraction, 70 mg of unfolded, young leaves were used. The plant material was dried overnight at 37 °C, subsequently frozen in liquid nitrogen and ground using a bead mill. The extraction was performed using the Nucleo Spin Plant II- Kit from Macherey and Nagel (Düren, D) following the manufacturer's instructions, with minor modifications. The concentration of genomic DNA was assessed spectrophotometrically at 260 nm and was evaluated for purity by determining the OD 260 nm/280 nm and OD

260 nm/230 nm ratios, respectively. The DNA quality was assessed by agarose gel electrophoresis.

AFLP® analysis

The AFLP® analysis was performed as described previously (Vos *et al.*, 1995), with minor modifications. For each sample, 100 ng of DNA was digested with *Hind*III and *Mse*I. The preamplification reactions were performed with specific primers that had an A as a selective base at the 3' end (*Hind*III (5'-AGACTGCGTACCAGCTT-A-3') and *Mse*I (5'-GACGATGAGTCCTGAGTAA-A-3')). *Hind*III (5'-AGACTGCGTACCAGCTT-ANN-3') primers with two selective bases and *Mse*I (5'-GACGATGAGTCCTGAGTAA-ANNN-3') primers with three selective bases were used for the final amplification. The *Hind*III primers were end-labelled either with the IRD 700 or IRD 800 dye (Eurofins MWG, Ebersberg, D). In total, 29 selective primer combinations were analysed (Table 3.1). The fragments were separated on 6 % polyacrylamide gels (Sequagel XR, Hessle, UK) using a DNA Analyzer (LI-COR, Lincoln, USA) and automatically documented using the e-Seq-Software (V3.0, LI-COR, Lincoln, USA).

Data analysis

The AFLP® banding patterns for each genotype were visually scored as present (1), absent (0) or ambiguous (?). The Jaccard index was chosen to calculate the pairwise genetic similarity between the genotypes. The similarities were transformed to distances by the formula $d = 1 - s$, where d is distance and s the Jaccard similarity. A cluster analysis was performed using the Neighbour-Joining method (Saitou and Nei, 1987). To evaluate the robustness of the dendrogram, a bootstrap analysis (Felsenstein, 1985) with 1000 replicates was conducted. The dendrogram was constructed using FAMD (Schlüter and Harris, 2006) and displayed with FigTree (Morariu *et al.*, 2008). A network was constructed using the Jaccard indices as the input for the Neighbour-Net algorithm of SplitsTree (Version 4.10; Huson and Bryant, 2006).

The FAMD was also used to estimate the Euclidean distance between the pairwise OUTs for the principal coordinate analysis (PCoA) via DARwin (Perrier and Jacquemoud-Collet, 2006).

Table 3.1: Primer combinations used for the AFLP® analysis. Only the selective bases are listed in the table below. The framework of the selective HindIII primers was 5'-AGACTGCGTACCAGCTT-NNN-3' and for the selective MseI primers was 5'-GACGATGAGTCCTGAGTAA-NNNN-3', respectively.

***Hind*III- IRD 700**	***Hind*III- IRD 800**	***Mse*I**
AAC	ATA	AAGC
AAC	-	ACAG
AAC	AAG	AAGG
AAC	AAG	ACCT
AAC	AAG	AGAG
AAC	AAG	ACAC
AAC	ACA	AGTC
ACGT	ACA	AGAG
AAC	ACA	ACCT
ACGT	ACA	ACCT
AGC	AAA	ACAC
AGC	AAA	ACGG
AGC	ATT	ACCT
AGC	ATT	AAGC
AGC	ATT	AAGG

The genetic structure was analysed using the 'admixture model' of *structure* 2.3.2.1 for dominant markers (ploidy level of six), with a burn-in period of 100000, followed by 300000 iterations. Seven independent runs were accomplished for each number of subpopulations (K), ranging from 1 to 12. The appropriate value for K was estimated as described by Pritchard *et al.* (2000), Cockram *et al.*, (2008) and as a function of the second order of change, as described in Evanno *et al.* (2005). The calculations and graph construction were conducted using R 2.13.1 (R Development Core Team, 2011).

To estimate the reliability of the genetic similarity data in relation to the number of markers used, a bootstrap analysis was performed using the R software. All of the pairwise genetic similarities (Jaccard) among the various marker sizes of 50, 100, 200, 300, 400 and 440 were calculated in 100 independent runs. The mean coefficient of variation (CV) was computed for every pairwise distance of the bootstrap analysis and plotted against the marker number. An example for the procedure using a marker size of 50 is given in the supplemental S3.2.

Results

Molecular data for the investigation of the chrysanthemum gene pool

For the 81 genotypes, a total of 448 polymorphic AFLP® markers were collected from 29 AFLP® primer combinations. On average, each AFLP® primer combination produced 15 polymorphic fragments across our set of genotypes. Only bands that were present or absent in at least two of the genotypes were scored.

One major advantage of AFLP® markers is their reproducibility when the method is properly applied (van de Jones *et al.*, 1997). To validate this, the AFLP® procedure was applied to four different chrysanthemum varieties in three independent replicates each starting from the harvest of leaves for DNA isolation. Four AFLP® primer combinations were used, resulting in an average of 72 marker fragments per primer combination. In all three replicates, the banding patterns for each of the four primer combinations were identical, indicating that the AFLP® patterns were reproducible under our experimental conditions.

Genetic diversity within the chrysanthemum gene pool is rather low

A 1/0 matrix of the polymorphic AFLP® fragments was used to compute the genetic similarity based on the Jaccard index. The relative genetic similarity within the germplasm ranged from 0.28 between the wild relative Cc1 and the cut-chrysanthemum 18060 to 0.9 between the outdoor-cut chrysanthemums OMR and OGL (Figure 3.1). The average genetic similarity was 0.69, with 50 % of the values ranging between 0.68 and 0.76. In Figure

3.1 there is a second peak with a maximum at 0.36. These low similarities are mostly comprised by the pairwise comparison of the *C. indicum* hybrid cultivars with the wild species (Cc1, HA2-39 and F-236). Without these species the genetic similarity ranged from 0.37 (between BREL and 18084) to 0.9 and the average similarity was 0.72. Since the genetic similarity is rather high, the genetic diversity is accordingly moderate within the *C. indicum* germplasm.

As we were mainly interested into the genetic diversity within the pool of *C. indicum* hybrid varieties, we excluded the three wild species (Cc1, HA2-39 and F-236) from our further analyses.

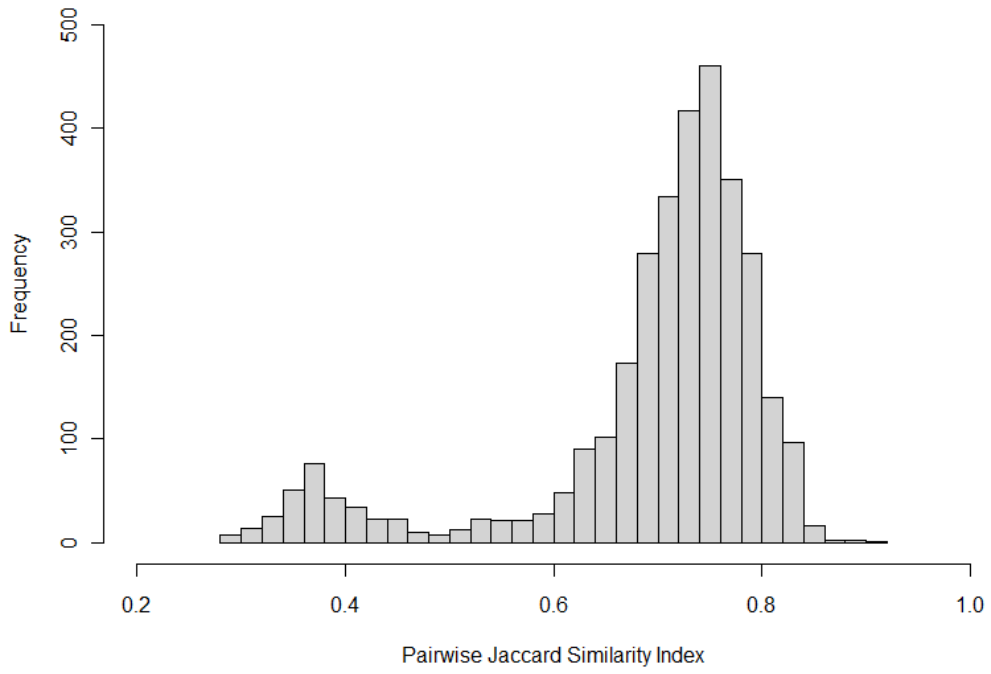

Figure 3.1: Frequency distribution of all of the pairwise similarity according to the Jaccard index, as based on the analysis of 448 AFLP® markers for 81 genotypes.

The Neighbour-Joining clustering resulted in mostly short branches, indicating high genetic similarities between most of the genotypes in accordance with the high frequency of the rather high Jaccard indices (Figure *3.2*a). The genotypes did not cluster into well separated groups as exemplified by the low bootstrap values (Figure 3.2b). Only a few varieties (e.g., BREL, 10130, 15285) belonged to a subcluster separated by bootstrap values above 50 %. However these varieties were cut (BREL and 10130) as

well as pot chrysanthemums (15285) from different breeders and displayed a high diversity for important phenotypic traits (e.g., growth rate, flower size, flower colour, branching rate; see supplemental Table S3.1). Nevertheless, some of the varieties were grouped pairwise together with high bootstrap values, such as HEWE and 8215 or 2510 and 5093. These represented varieties of the same breeding line, respectively. A recomputation of the phenograms based on alternative distance indices, for example, Nei and Li (Nei and Li, 1979) or other clustering methods, such as UPGMA, resulted in similar dendrogram topologies, with the same lack of bootstrap support (data not shown). These methods could not resolve any structure corresponding to their origin (breeding company) or important phenotypic traits.

Bootstrap analysis supports marker saturation

The low statistical support of the clusters might be due to an insufficient number of markers. Therefore, we computed the Jaccard similarities for all of the pairs of genotypes by repeated, random sampling of different marker numbers (50, 100, 200, 300, 400 and 440 markers, each randomly sampled 100 times) and estimated the coefficient of variation (CV) of the Jaccard indices (Figure 3.3). The variation decreased rapidly with an increasing number of markers. By using only 100 markers, the mean CV was already significantly less than 10 %. The largest CVs were obtained for pairs of genotypes having the highest similarities. Using 440 markers, the coefficient of variation fell below 10 % for all of the pairwise similarities. Therefore, the number of tested markers appeared to be sufficient for a precise estimate of the genetic variation in this group of 78 chrysanthemum genotypes.

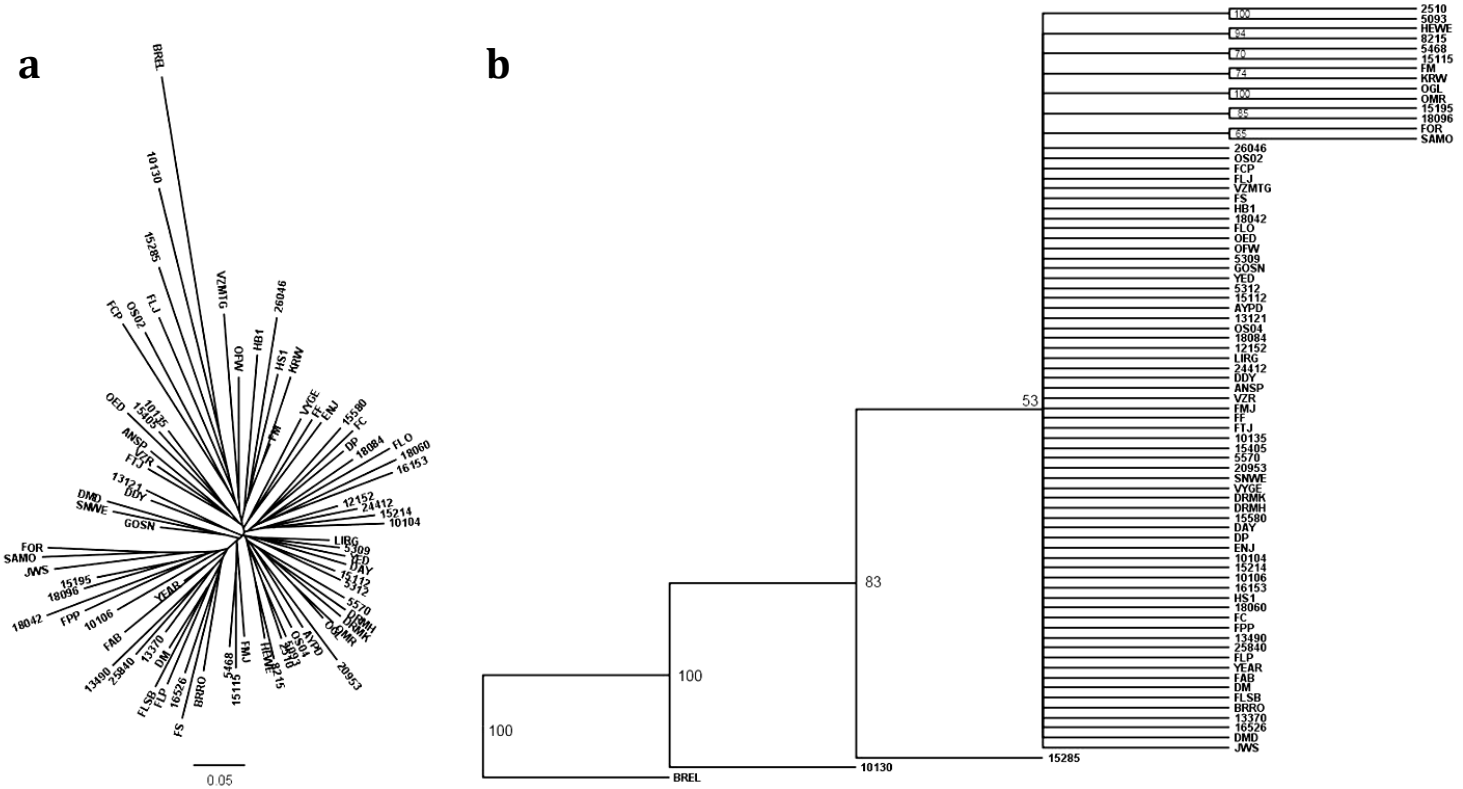

Figure 3.2: a) Relatedness of 76 *C. indicum* varieties and 2 related chrysanthemum species by Neighbour-Joining clustering using 448 polymorphic AFLP® markers. The branch length scales the genetic distances according to the Jaccard index. b) Majority-rule consensus tree obtained from 1000 bootstrap replicates. The bootstrap values above 50 % are shown beside the nodes.

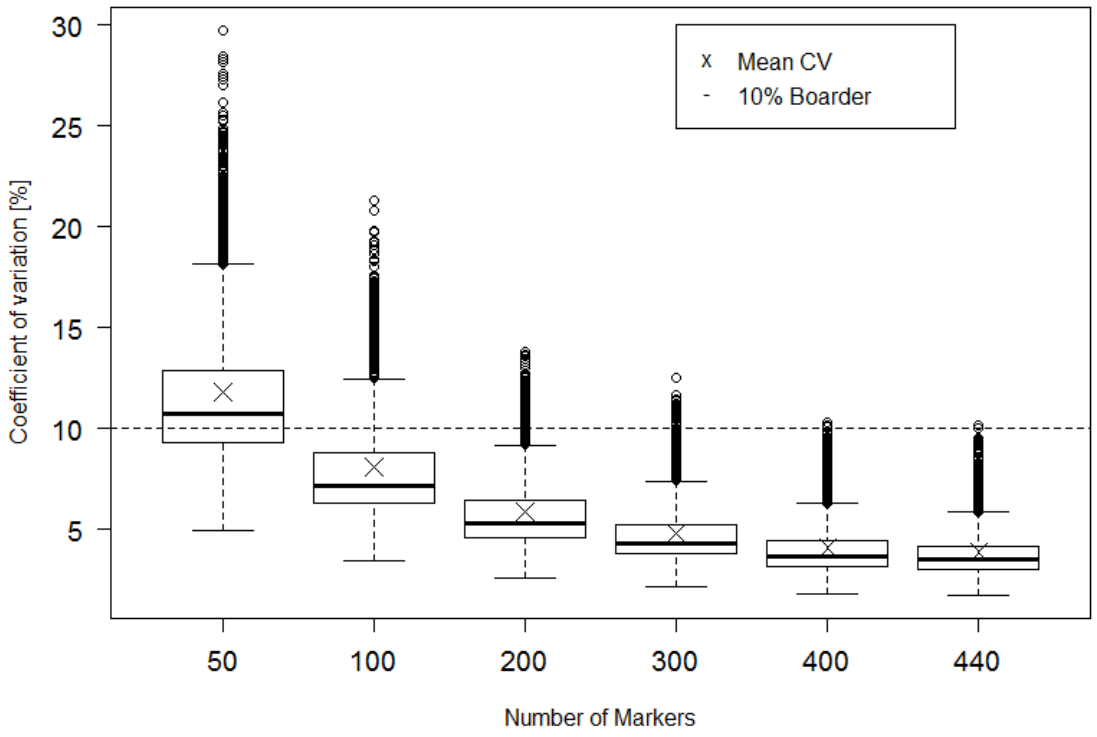

Figure 3.3: Mean coefficient of variation (CV) of the genetic similarities for all of the pairwise Jaccard indices for 100 randomly replicated AFLP® marker sets with varying marker numbers. The boxes represent the 25 and 75 quartiles, the whisker caps indicate the 10 and 90 percentiles, the medians are indicated by the bold line, and the mean is represented by x. The dotted line indicates a CV of 10 %.

Other phylogenetic methods lack a structure in the chrysanthemum gene pool

As the construction of a dendrogram is based on the assumption of bifurcating evolution of the taxonomic units, such events as hybridisation, recombination or backcrossing will introduce errors into the pairwise clustering. Therefore, we used other concepts, including PCoA, networks and *structure*, to decipher other possible genetic relationships within the germplasm.

The associations among the genotypes revealed by PCoA are shown in Figure *3.4*. The first two axes explained approximately 12 % of the total variation, with 7.3 and 5.1 % for the first and second axis, respectively. The first 14 coordinates had positive eigenvalues above 1. Most of the genotypes were scattered around the negative part of the x-axis of the graph. Two other clusters are visible in sector 1 and 2, respectively. There was no further grouping according to the breeders, classes (cut, pot or garden) or morphological traits within any of these clusters.

Only the *C. indicum* hybrid varieties BREL, 10130 and FLJ were well separated from all other accessions.

Hardly any differentiation into groups was also present when we computed the Neighbour-Net network (data not shown).

The estimated number of populations (K) based on the *structure* analysis seemed to be between one and two for the analysis according to Pritchard *et al.* (2000) and also for the method according to Cockram *et al.* (2008) (see supplemental S3.3). These findings are in agreement to the results of the analyses before. However, if we applied the ΔK method of Evanno *et al.* (2005), the most likely K was nine (see supplemental S3.3). Nevertheless, the ΔK method is only able to detect a K of at least two and the grouping of the genotypes to the obtained clusters was only conserved for some of the investigated varieties between the seven repeated runs.

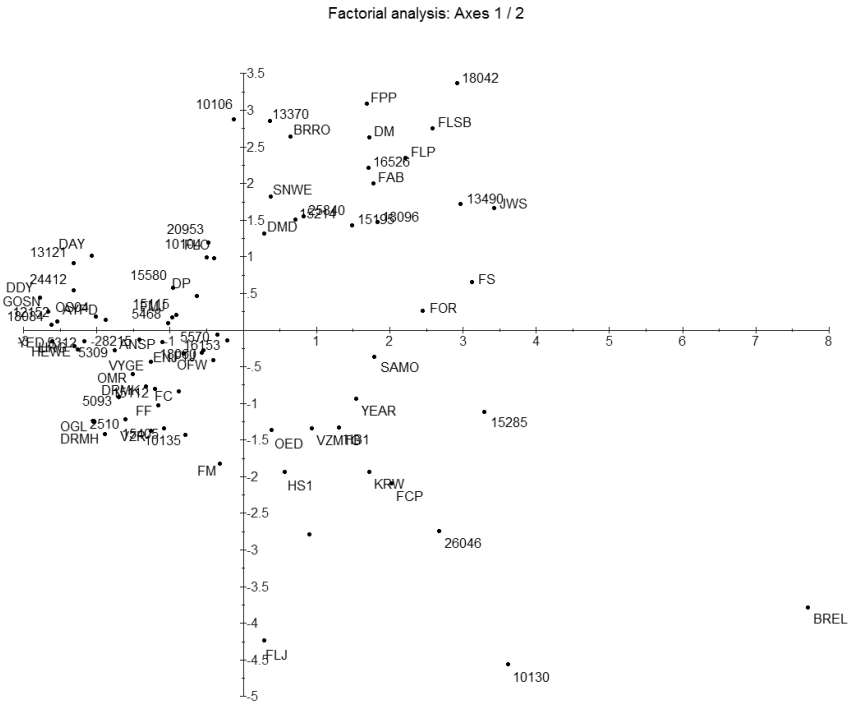

Figure 3.4: Plot showing the first two axes of a principal coordinate analysis (PCoA) based on AFLP® data depicting the genetic relationship by the Euclidean distance among the chrysanthemum genotypes without wild species. The *C. indicum* hybrid varieties BREL, 10130 and FLJ are most distinct from most of the other accessions.

Discussion

We analysed the genetic similarity within a collection of different *C. indicum* hybrid varieties and some related wild species (*C. coccineum, C. maximum, C. haradijanii* and *C. x hortorum*). Most of the (European) varieties were genetically close to each other, as exemplified by 75 % of all of the pairwise genetic similarities ranging from 0.6 to 0.9, with an average of 0.72. However, this moderate or rather high genetic similarity is in contrast to a large phenotypic diversity of the varieties for the major traits, such as growth, branching rate, flower size or colour (see supplemental Table S3.1). This phenotypic diversity would be expected for an outcrossing, self-incompatible and polyploid ornamental, which should be highly heterozygous (Drewlow *et al.*, 1973).

Indeed, the investigated phenotypes do not cluster according to these phenotypic traits or the breeding programmes of different breeders. In a comparable study by Wegner and Debener in 2008 using dahlia cultivars, a similar lack of correlation between different horticultural groups, as based on their inflorescence morphologies and UPGMA clustering, was observed. As horticultural characteristics represent only a small section of the chrysanthemum genome, the weight of these factors is low in comparison to the AFLP® data, which reveal the entire genetic background. Thus, the resulting tree in this investigation shows a discrepancy between the morphological and genetic classification.

Although there seems to be a lack of structure in the gene pool of chrysanthemum, as the Neighbour-Joining dendrogram is little supported by the bootstrap values in our study, some genotypes, such as HEWE and 8215 or 2510 and 5093, are grouped together with high bootstrap values. Both pairs of varieties were developed by the same breeder and are likely to be related by descent. Therefore, although we were able to detect such a close relationship in our study, most of the investigated genotypes lack such a supported structure.

There are possible reasons for the low support via the bootstrap analysis. A crucial point for diversity estimation is the number of polymorphic molecular markers because larger numbers of them provide more-precise estimates of the genetic relationship (Tivang *et al.*, 1994). We determined the sampling variance of the molecular marker data set by the application of the bootstrap procedure. These

results indicate that the 448 AFLP® markers used in this study achieved a CV less than 10 % in the estimation of the genetic distance among the 78 chrysanthemum genotypes. As a CV value below 10 % is considered to be necessary to achieve precise genetic distance estimates (Tivang *et al.*, 1994; Thormann *et al.*, 1994), the marker number is not likely to be the reason for the low bootstrap support.

However, the bootstrap procedure itself has limitations (for a detailed examination, see Soltis and Soltis, 2003). For example, the bootstrap support is affected by the taxon sample size and decreases with an increasing number of samples (Sanderson and Wojciechowski, 2000). Another problem often found in closely related species that have not diverged extensively is that even nonconflicting groups that are only supported by a few characters might result in low bootstrap values. In these cases, the addition of markers that are not informative or autapomorphic for a specific node could decrease the bootstrap value for this node (Soltis and Soltis, 2003). In our germplasm sample, most of the genotypes share a rather high similarity, with an average genetic similarity of 0.69. Therefore, it is likely that the varieties from the different breeders were intercrossed and backcrossed to generate new varieties. As a result, modern varieties are closely related and have not diverged extensively, which explains the low bootstrap support of the clustering, despite the low CV in the distance estimates. The underlying genetic relationships among the genotypes might then be more network-like then a dendrogram with separate clusters.

Other methods not relying on bifurcating clustering methods, such as PCoA, networks or population structure, also placed all of the varieties into one large unstructured group and only separated the wild *Chrysanthemum* species (HA2-39, Cc1 and F-236) and the *C. indicum* hybrid varieties BREL, 10130 and 15285 from this group. This result supports a close relationship and admixture of the cultivated germplasm, a situation that most likely cannot be completely resolved using molecular markers, even in such large numbers.

We found a range of the genetic similarity from 0.37 to 0.9 in the chrysanthemum varieties studied. Regarding other cultivar groups belonging to the *Asteraceae*, the similarity values varied from 0.909 to 0.9995 for *Osteospermum* and *Dimorphotheca* (Gawenda and Debener, 2009), 0.58 to 0.93 for *Dahlia*

(Wegner and Debener, 2008) and 0.294 to 0.958 for *Gerbera hybrida* (Gong and Deng, 2012). Although the genetic similarity values for *Osteospermum* and *Dimorphotheca* were higher, Gawenda and Debener (2009) were able to detect four major and two minor clusters with bootstrap support. Conversely, for the dahlia and gerbera cultivars, with a comparable genetic similarity as chrysanthemum, a similar lack of clearly supported clusters with bootstrap values above 50 % was observed (Wegner and Debener, 2008; Gong and Deng, 2012). These results may indicate that the lack of bootstrap support is not due to a high genetic similarity or an insufficient number of markers but is rather influenced by the breeding history of the cultivars. *Osteospermum* cultivars belong to the so-called "new ornamental" plants. because this group includes a limited number of varieties (< 200) having CVPO Granted Community Plant Variety Rights (http://www.cpvoextranet.cpvo.europa.eu/WD150AWP/WD150AWP.exe/CONNECT/ClientExtranet), their breeding history is most likely much less complex, with less backcrosses and less repeated use of the same genotypes in different breeding companies compared to the "old ornamental" chrysanthemum with more than 1000 CVPO granted varieties. This circumstance led to a less-complex kinship structure in *Osteospermum*, which is easier to resolve with molecular markers.

In summary, we did not detect any structure within the group of chrysanthemum cultivars. The overall genetic similarities between the cultivars were comparatively moderate to high. However, with the low variability in our similarity estimates, our data might be used to select parents for experimental segregating populations in which both the degree of phenotypic and marker polymorphism is maximised with respect to unselected parental combinations. This would significantly facilitate the genetic analyses of many important ornamental traits in chrysanthemum and, therefore, improve the targeted breeding strategies supported by molecular markers.

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4 Allelic variants of strigolactone pathway genes shape plant architecture: a case study on the inheritance of horticultural traits in chrysanthemum

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Abstract

Background

Shoot branching is crucial for the aesthetic value of a plant. Chrysanthemums (*Chrysanthemum indicum* hybrid) are important ornamental plants with abundant phenotypic diversity; however, very little is currently known about the inheritance of horticultural traits or corresponding marker-trait associations.

Results

We phenotyped and genotyped two types of chrysanthemum populations: a collection of 81 varieties and a biparental F1 population of 160 individuals. We identified 15 marker-trait associations with AFLP® markers for the genotype collection using a genome-wide association study and 17 marker-trait associations for the population by applying a single locus analysis. Additionally, a candidate gene approach for strigolactone pathway genes identified marker alleles that were significantly associated with shoot branching in both populations. These genes described a large proportion of the variation in shoot branching in these populations.

Conclusions

This study highlights the fundamental role of the strigolactone pathway and indicates that shoot branching in the chrysanthemum has a polygenic inheritance pattern, though other yet unknown factors are also likely involved. Although

nearly all of the investigated traits were characterised by a continuous variation in phenotypic values, as was expected for the outcrossing hexasomic nature of the chrysanthemum, we identified informative marker-trait associations with important characteristics.

Key words

chrysanthemum, genome wide association study, MAX pathway, molecular marker, QTL, shoot branching, strigolactone

Background

Chrysanthemums (*Chrysanthemum indicum* hybrid, *C.* x *grandiflorum* or *C. morifolium*) are one of the most economically important ornamental plants worldwide, surpassed only by roses. The success of this plant is based on its abundant diversity of flower types, colours and plant architectures (Zhang *et al.*, 2010), as these traits satisfy the continuous demand for novel phenotypes in ornamental plant production. Although the application of molecular markers has improved plant breeding in important crops such as maize, rice, soybeans or tomatoes (as reviewed by Babu *et al.*, 2004), very few studies have developed molecular markers for important horticultural traits in chrysanthemums in particular and ornamentals in general (Zhoa *et al.*, 2009; Zhang *et al.*, 2011a; Zhang *et al.*, 2011b).

Molecular analyses of cultivated chrysanthemums are hampered by the complex genetics of this species. As an out-crossing and self-incompatible ornamental (Drewlow *et al.*, 1973), cultivated chrysanthemums are highly heterozygous. They are generally believed to be the result of natural hybridisations of species such as *C. indicum* L., *C. morifolium* and others such as *C. vestitum* or *C. lavandulifolium* (Vogelmann, 1969; Dai *et al.*, 1998; Yang *et al.*, 2006); these hybridisations lead to the formation of segmental allohexaploids with 54 chromosomes (Dowrick, 1953; Klie unpublished observations). Understanding how important horticultural characteristics such as the diameter of inflorescences, plant height or shoot branching are inherited and identifying loci for candidate genes contributing to the phenotypic expression of these traits is of great interest. Therefore, we combined an association mapping approach in a collection of chrysanthemum varieties (Klie *et al.*, 2013) with quantitative trait locus (QTL) detection in a biparental F1

population using amplified fragment length polymorphism (AFLP®) markers and markers derived from candidate genes. Both methods have advantages and disadvantages. While association mapping produces maps with a higher resolution and can potentially detect a higher number of alleles and uses a broader reference population, linkage mapping has more power to detect low-effect QTL for a given trait and is not affected by the population structure (Stich and Melchinger, 2010).

Because the genetic control of shoot branching was of special interest, we used anonymous multilocus AFLP® markers and included candidate genes of the strigolactone (SL) pathway and the transcription factor *BRANCHED*1 (*BRC*1) in our analysis. SL was recently identified as a phytohormone that regulates bud outgrowth and side shoot formation (Gomez-Roldan *et al.*, 2008; Umehara *et al.*, 2008); it is derived from carotenoid precursors that are enzymatically processed (e.g., by CCD7, CCD8, a phytochrome P450 and others) and its activity is dependent on recognition by the F-box receptor *MAX*2. The outgrowth of buds is controlled by SL and its interaction with the phytohormones auxin and cytokinin (CK). On the one hand, SL reduces the capacity of the polar auxin transport stream (PAT) in the main stem by altering polar auxin transport, which leads to enhanced competition between buds to release their auxin into the stem and increased outgrowth (Crawford *et al.*, 2010; Shinohara *et al.*, 2013). On the other hand, SL and CK are transported acropetally through the xylem and directly control the outgrowth of the buds by jointly regulating the TCP transcription factor *BRC*1 (Braun *et al.*, 2012; Dun *et al.*, 2013). The genes encoding *CCD*8 (Liang *et al.*, 2010), *MAX*2 (Dong *et al.*, 2013) and *BRC*1 (Chen *et al.*, 2013) have been functionally characterised in the chrysanthemum.

Not all components of the strigolactone biosynthesis and perception pathway have been identified in the chrysanthemum, and it is unclear whether allelic variants of these genes are associated with phenotypic variance in shoot branching. Our aim was to identify allelic variants for the characterised genes *CCD*8, *MAX*2, *BRC*1 and the uncharacterised gene *CCD*7 by single-strand conformation polymorphism (SSCP) analysis in different populations of chrysanthemum. Furthermore, we were interested in characterising the inheritance pattern of shoot branching and other important ornamental traits and

therefore sought to identify yet uncharacterised loci for candidate genes using AFLP® markers in an association study and in QTL detection.

Methods

Plant material

We used 81 chrysanthemum genotypes from various breeding companies; in particular, 76 varieties of *Chrysanthemum indicum* hybrids and five accessions of four previously described species (Klie *et al.*, 2013) were used. Three clones of each genotype were cultivated in 7.5-L pots with a fertilised substrate ("Einheitserde T") in a greenhouse under a 16 h light/8 h dark cycle at a constant temperature of 22 °C. After four weeks, the cycle was changed to 8 h light/16 h dark to induce flowering. Fully opened flowers were phenotyped. Data on the other morphological traits (plant height, number of nodes, number of axillary buds, number of formed side shoots, length of the internode and length of the longest side shoot) were collected for all three clones of the 81 genotypes after senescence of the flowers.

We established a segregating biparental F1 population (MK11/3) of 160 individuals by crossing the female parent *C. grandiflorum* 'Kitam' (541) with the paternal parent 'Relinda' (VZR), a registered *C. indicum* hybrid variety. One clone of each genotype was cultivated in three independent randomised blocks with 48 cuttings per m^2 in 12.5 cm × 12.5 cm plots. The plants were grown in a fertilised substrate that consisted of a mixture of peat and chalked compost soil in a greenhouse under a 16 h light/8 h dark cycle at a constant temperature of 22 °C from week 34 until week 38. Subsequently, the cycle was changed to 8 h light/16 h dark to induce flowering. Flowers were phenotyped between weeks 45 and 47. Data on the other morphological traits (plant height, number of nodes, number of formed side shoots, and length of the longest side shoot) were collected for all three clones of the 160 genotypes after senescence of the flowers.

DNA extraction

For DNA extraction, 70 mg of unfolded young leaves were dried overnight at 37 °C, frozen in liquid nitrogen and ground using a bead mill. The extraction was performed using the NucleoSpin Plant II Kit from Macherey and Nagel (Düren, D)

following the manufacturer's instructions, with minor modifications. The concentration of genomic DNA was assessed spectrophotometrically at 260 nm and evaluated for purity by determining the OD 260 nm/280 nm and OD 260 nm/230 nm ratios. DNA quality was assessed by agarose gel electrophoresis.

Marker analysis

AFLP® analysis

The AFLP® analysis was performed as described previously (Vos *et al.*, 1995), with minor modifications. For each sample, 100 ng of DNA were digested with 9 U *Hind*III and 3.5 U *Mse*I. The preamplification reactions were performed with specific primers that had an A as a selective base at the 3' end (*Hind*III (5'-AGACTGCGTACCAGCTT-A-3') and *Mse*I (5'-GACGATGAGTCCTGAGTAA-A-3')). *Hind*III (5'-AGACTGCGTACCAGCTT-ANN-3') primers with two extra selective bases and *Mse*I (5'-GACGATGAGTCCTGAGTAA-ANNN-3') primers with three extra selective bases were used for the final amplification. The *Hind*III primers were end-labelled with either the IRD 700 or IRD 800 dye (Eurofins MWG, Ebersberg, D). A total of 64 and 21 selective primer combinations were analysed in the chrysanthemum genotype collection and MK11/3 population, respectively. The fragments were separated on 6 % polyacrylamide gels (Sequagel XR, Hessle, UK) using a DNA Analyzer (LI-COR, Lincoln, USA) and automatically documented using the e-Seq-Software (V3.0, LI-COR, Lincoln, USA).

SSCP markers for candidate genes

Candidate genes for shoot branching (*CCD*7, *CCD*8, *MAX*2 and *BRC*1) were screened for polymorphisms using single-strand conformation polymorphism (SSCP) analysis. Several primer pairs were used for each candidate gene, and different fragments of the entire sequence of each gene were amplified (Table 4.1). Most of the PCR products were labelled at the 5' end with infrared (IRD)-dyes using universal M13 sequences (5'-GTAAAACGACGGCCAGT-3' for forward and 5'-CAGGAAACAGCTATGAC-3' for reverse primers) under the following PCR conditions (Schuelke, 2000): 0.2 µM of each non M13-tailed primer, 0.06 µM M13-tailed primer, 0.07 µM M13 primer end-labelled with the IRD 700 dye (Eurofins MWG, Ebersberg, D) in a final 25 µL reaction volume (2x Williams Buffer, 0.16 mM dNTPs, 0.7 U DCS-Taq polymerase [Enzymatics, Beverly, USA] and 30 ng template

DNA). Conditions for the PCR amplification were as follows: 95 °C for 3 min, 25 cycles at 94 °C (30 s) / 58 °C (30 s) / 72 °C (45 s) followed by eight cycles of 94 °C (30 s) / 52 °C (45 s) / 72 °C (60 s) and a final extension at 72 °C for 10 min. All other PCR products were amplified with a standard PCR in a 20 µL reaction volume composed of 1 x Williams Buffer, 0.2 mM dNTPs, 0.5 µM primers, 0.5 U DCS Taq polymerase and 30 ng of template DNA. Conditions for the PCR amplification were as follows: 95 °C for 3 min, 30 cycles at 94 °C (30 s) / 60 °C (60 s) /72 °C (60 s) followed by a final extension at 72 °C for 10 min. An equal volume of SSCP dye (95 % formamide, 0.01 M NaOH, 0.05 % xylene cyanol, 0.05 % bromophenol blue) was added to each PCR reaction; the samples were then denatured for three minutes at 95 °C. The denatured samples were immediately cooled on ice (10 °C) and loaded on 0.5 x MDE gels (0.5x MDE® gel solution [Lonza Group Ltd, Basel, SUI], 0.6x long run TBE [80.4 mM Tris, 7.5 mM Borsäure, and 1.5 mM EDTA], 8.3 % glycerine, 0.05 % APS, 10 µL TEMED, *ad* 15 mL water). IRD-labelled single-strands were detected on an Odyssey® Infrared Imaging System (LI-COR, Lincoln, USA) and automatically documented using the Odyssey software (V3.0, LI-COR, Lincoln, USA). Non IRD-labelled single-strands were visualised by silver staining according to the protocol of Sanguinetti *et al*. (1994).

Data analysis

The marker banding patterns for each genotype were visually scored as present (1), absent (0) or ambiguous (?/NA).

The genetic structure of the collection of genotypes used for association mapping was analysed previously (Klie *et al.*, 2013). Therefore, assuming two subpopulations, a Q-matrix was computed with the software *structure* 2.3.2.1 (Pritchard *et al.*, 2000) and used as a covariate for the association studies. A kinship-matrix was estimated for 1000 AFLP® markers according to the method of (Hardy, 2003) with the SPAGeDi software (Hardy and Vekemans, 2002) and used as a second covariate. The mixed linear model (Zhang *et al.*, 2009) of TASSEL version 3.0 (Bradbury *et al.*, 2007) was used with both covariates (population structure Q and kinship-matrix K) to calculate marker associations for the 1000 AFLP® markers and each candidate gene, respectively. Only markers with an allele-frequency of more than 5 % were allowed. Markers were considered significantly

associated with the candidate genes for a significance level of $\propto = 0.05$ and after Bonferroni correction for the AFLP® markers (p-value × n marker, $n = 1000$).

QTL for the MK11/3 population were identified by analysis of variance (ANOVA) of the AFLP® markers and markers for the candidate genes. A significance level of $\propto = 0.05$ was used for the candidate genes. The p- and q-values of the AFLP® markers were calculated and ranked; markers with an additive q-value of less than 1 ($\sum q < 1$; Herrera and Bazaga, 2009) were considered significantly associated with the candidate gene. All calculations were conducted with the qvalue package of the R software version 2.15.2 (R Development Core Team, 2011).

The effect of the marker locus on the corresponding trait was estimated as the ratio of means for groups with and without the marker for almost all traits; the percentage of outgrowth was expressed as the difference of means.

Results

Phenotypic Data

Phenotypic data for the collection of chrysanthemum genotypes

We phenotyped a collection of 81 chrysanthemum genotypes for plant height, internode length, number of nodes, number of axillary buds, number of formed side shoots, length of the longest side shoot and flower diameter. All traits were measured in three clones of each genotype and treated as quantitative non-normally distributed parameters (see supplemental Figure S4.1). Plant heights ranged from 19.1 to 100.1 cm, the internode length ranged from 0.7 cm to 3.5 cm, the number of nodes ranged from 13 to 44, the number of formed axillary buds ranged from 9 to 41, the number of formed side shoots ranged from 6 to 31, the length of the longest side shoot ranged from 3.6 to 47.4 cm and the flower diameter ranged from 2 to 14 cm. To measure the branching propensity of the plant, we estimated the ratio of formed side shoots to the number of nodes; this parameter ranged from 16 to 82 %.

Table 4.1: List of primer pairs for the candidate genes *CCD*7, *CCD*8, *MAX*2 and *BRC*1 used in the single-strand conformation polymorphism (SSCP) analysis**.** Gene, GB accession, primer combination, primer sequences (5' - 3'), tested genetic resources (G) chrysanthemum germplasm, P) population MK11/3), size of expected PCR product and the detection method. Primers marked by an asterisk included a universal M13 sequence (5'-GTAAAACGACGGCCAGT-3' for forward and 5'-CAGGAAACAGCTATGAC-3' for reverse primers) at the 5' end for IRD-labelling of PCR fragments. These fragments were detected via infrared imaging, whereas non-labelled fragments were detected via silver staining.

Gene	Accession	PC	Primer Pairs		Resource	Product Size	Detection Method
CCD7	unpublished	A	F	CCCTCTAGATGGTCATGG	G+P	550 bp	silver staining
			R	AGCAAGATCTAACAAGTCCACACCAC			
		B	F*	TGTCATGCAACGCAGAGGAT	G+P	1750 bp	M13-IRD700
			R	CCCACATTTGAGAAGGAGCTT			
		C	F	GGTGGGGCCCCTTACGAGAT	G+P	600 bp	silver staining
			R	GCATTGCATGACATCATAAG			
		D	F*	TCCATGACTGGGCTTTCACA	P	380 bp	M13-IRD700
			R	CCCACATTTGAGAAGGAGCTT			
CCD8	Liang *et al.*, 2010	A	F*	ATGGCATCCTGAGTCGAAAG	G+P	550 bp	M13-IRD700
			R	GCGTCTACTAGTTCTCCCTTTGG			
		B	F*	ACAAGCTGCGGCTTCAAA	G+P	260 bp	M13-IRD700
			R	GCGTCTACTAGTTCTCCCTTTGG			
		C	F*	GGTGCGTCCCTAACTGACAA	G+P	480 bp	M13-IRD700
			R	GACTCAGGATGCCATTCAAAC			
		D	F	TAGCAAACCTCTTTATTACCGATGG	G	420 bp	M13-IRD700
			R*	TGGATATGAAGTGGTGCACTAGA			
		E	F	AACAAACAGCGGAGGTTAAAA	G	300 bp	M13-IRD700
			R*	GAAGTAGAGGCGGACCATGG			
MAX2	JX556222	A	F*	GCCAATCCAGGGTCGGATAC	G+P	550 bp	M13-IRD700
			R	GTAACGACAAACTCCTCTGG			
		B	F*	ATGTCTTTCTCCACCACAACAAT	G+P	1200 bp	M13-IRD700
			R	GCACCTAAATTCATACAACACGAG			
BRC1	JX870411	A	F	TGCAGCATCAGTTCAGTGACT	G+P	380	M13-IRD700
			R*	AGCAGTAGCATACAATTGACATAGT			

No strong correlations between phenotypic traits were observed (see supplemental Figure S4.2). The plant height was correlated with the length of the longest side shoot ($R^2 = 0.6$) and with the internode length ($R^2 = 0.45$). The number of nodes was weakly correlated with the number of formed side shoots ($R^2 = 0.28$).

Phenotypic data for the population MK11/3

The 160 progeny of the MK11/3 population were phenotyped for plant height, number of nodes, number of axillary buds, number of formed side shoots, length of the longest side shoot and inflorescence diameter. All traits were measured in three independent clones of each genotype, and all traits were normally distributed quantitative parameters (see supplemental Figure S4.3). The plant heights ranged from 21.5 to 65.8 cm, the number of nodes ranged from 15 to 35, the number of formed side shoots from 0 to 26, the length of the longest side shoot ranged from 0.5 to 19.4 cm and the flower diameter ranged from 3.2 to 8.5 cm. The ratio of formed side shoots to the number of nodes (percentage of outgrown side shoots) ranged from 0 to 1.

No strong correlations between phenotypic traits were detected (see supplemental Figure S4.4). The plant height was weakly correlated with the length of the longest side shoot ($R^2 = 0.24$). The number of nodes was weakly correlated with the number of formed side shoots ($R^2 = 0.22$).

Genome wide association study of the chrysanthemum genotypes

We used 64 selective primer combinations to generate 1000 polymorphic AFLP®-bands for the collection of 81 chrysanthemum genotypes. These marker data were used to identify genomic regions that contribute to the phenotypic variance of the traits in a genome wide association study (GWAS). Applying the mixed linear model (MLM) of TASSEL with a Q-Matrix of *structure* and a kinship matrix from the SPAGeDi software as covariates, 15 AFLP® markers were determined to be significantly associated ($\propto = 0.05$) with phenotypic traits after Bonferroni adjustment of the p-values. The MLM approach revealed that one marker was associated with the percentage of outgrown side shoots, another

marker was associated with the number of formed side shoots, four markers were associated with the length of the longest side shoot, one marker was associated with the ratio of plant height to length of the side shoot, seven markers were associated with the flower size and one marker was associated with the length of the internode (see Table 4.2).

Table 4.2: Markers associated with several phenotypic traits as revealed by a genome-wide association study (GWAS) in a collection of chrysanthemum genotypes. The marker effect is given as the ratio of means for groups with and without the marker; the percentage of outgrowth is given by the difference in means. Bonferroni-adjusted p-values (p-value × n marker, $n = 1000$) are shown with a significance level of $\propto = 0.05$.* log of the trait

Trait	**Marker**	**Effect**	**Bonferroni adjusted p-value**
Percentage of outgrown side shoots	HAGC_MACCT_112	-16 %	0.01
Number of formed side shoots	HAAC_MAAGG_17*	+25 %	0.002
Length of the longest side shoot	HACGT_MAGAG_48	+23 %	$1.09e^{-9}$
	H7AGC_MACAT_340	-17 %	$3.30e^{-9}$
	HACA_MAGAG_107	-11 %	$2.75e^{-6}$
	HAAA_MACGG_53	+12 %	$7.38e^{-5}$
Ratio of plant height to length of the side shoot	HAAG_MACCT_135	-234 %	0.008
Flower Size	HAGT_MATTA_68	+2 %	$4.50e^{-4}$
	HAGT_MATTA_94	+15 %	$6.01e^{-4}$
	HAGT_MATTA_110	+7 %	$8.77e^{-4}$
	HAGT_MATTA_130	+21 %	0.001
	HAGT_MATTA_140	+20 %	0.001
	HAGT_MATTA_210	+31 %	0.001
	HATT_MACCT_124	+13 %	0.023
Length of the internode	H7AGT_MACCT2_6	-10 %	0.018

The presence of marker HAGC_MACCT_112 resulted in a 16 % mean decrease in the outgrowth of side shoots. Plants carrying the marker fragment

HAAC_MAAGG_17 formed 25 % more side shoots than plants without the marker. The length of the longest side shoot was affected by four different AFLP® markers; these markers caused variations in shoot lengths of -11 % to +23 %. The HAAG_MACCT_135 marker resulted in a 234 % decrease in the ratio of plant height to shoot length, but only three plants with a large ratio did not have this fragment. Flower size was influenced by seven markers, resulting in variations in flower size of 2 to 31 %. The H7AGT_MACCT2_6 marker decreased the mean length of the internode by 10 %.

QTL detection in the MK11/3 population

Marker data were generated from 21 AFLP® primer combinations for the MK11/3 population. A total of 327 markers were scored and transferred into a 1/0 matrix. We attempted to calculate linkage maps with the help of single-dose markers for both parents but were unable to because of a relatively restricted number of markers and the hexaploid nature of *Chrysanthemum*. Therefore, we analysed our data one AFLP® marker at a time to identify quantitative trait loci (QTL) via ANOVA. For the AFLP® markers, p-values were calculated; subsequently, q-values were calculated and ranked. We identified 17 markers that had an additive q-value of less than 1 ($\sum q < 1$; Herrera and Bazaga, 2009) and were significantly associated with various traits. The QTL detection identified three markers associated with the percentage of outgrown side shoots (with variations ranging from -10 to 7 %), one marker associated with the number of nodes, three markers associated with the number of formed side shoots (with variations ranging from -23 to -22 %), one marker for both plant height and for the length of the longest side shoot (most likely affecting the length of the internode), one marker for the ratio of plant height to length of the side shoot and seven markers for flower size (see Table 4.3).

The marker HACG_MACCT_13 was associated with a mean decrease of 7 % in the number of nodes. Plants with the marker fragment HAAT_MAAGG_23 were 13 % taller and had side shoots that were 27 % longer than plants without the marker. The marker HACA_MACAT_1 decreased the ratio of plant height to the length of the longest side shoot by 34 %. Flower size was influenced by seven markers and varied by -13 to 11 %.

Table 4.3: QTL for several phenotypic traits in the MK11/3 population as calculated by ANOVA. The marker effect is given as the ratio of means for groups with and without the marker and the percentage of outgrowth is given as the difference in means. In addition to the p-value, the q-value is given for each marker to control for the effect of multiple testing of various markers in the ANOVA. All markers were ranked according to their q-value and accepted, resulting in an expectation of less than one falsely significant association ($\Sigma q < 1$;, see Herrera and Bazaga, 2009).

Trait	**Marker**	**Effect**	**p-value**	$\sum q < 1$
Percentage of outgrown side shoots	HAAT_MACGA_6	-10 %	0.002	0.235
	HACA_MACAG_1	+7 %	0.002	0.235
	HAGT_MACAT_11	-7 %	0.003	0.273
Number of nodes	HACG_MACCT_13	-7 %	0.002	0.591
Number of formed side shoots	HAAT_MACGA _6	-23 %	0.002	0.261
	HAGA_MACGA_1	-22 %	0.002	0.261
	HAAT_MACGA _25	-23 %	0.003	0.261
Plant height	HAAT_MAAGG_23	+13 %	0.003	0.555
Length of the longest side shoot	HAAT_MAAGG_23	+27 %	0.003	0.622
Ratio of plant height to length of the side shoot	HACA_MACAT_1	-34 %	0.012	0.76
Flower Size	HAGA_MACGA_13	+11 %	$7.79e^{-06}$	0.001
	HACG_MACGA_12	+10 %	$6.45e^{-04}$	0.044
	HACG_MATCA_17	-11 %	$6.40e^{-04}$	0.044
	HAGT_MAGCA_1	-13 %	0.002	0.050
	HAGC_MACCG_2	-11 %	0.006	0.074
	HACG_MAAGC_2	+9 %	0.007	0.198
	HATG_MATGG_2	-11 %	0.006	0.198

Analysis of candidate genes of the strigolactone pathway

In addition to a general investigation into the inheritance of phenotypic traits, we also investigated the genetics of shoot branching in the chrysanthemum (Figure 4.1a, b). We applied a candidate gene approach based on genes known to affect shoot branching in other plant species. We chose the so-called *MAX*-pathway genes (*CCD*7, *CCD*8, *MAX*2) and *BRC*1 as candidate genes because these genes have been

shown to be associated with the control of shoot outgrowth via the phytohormone strigolactone in the chrysanthemum and other plants.

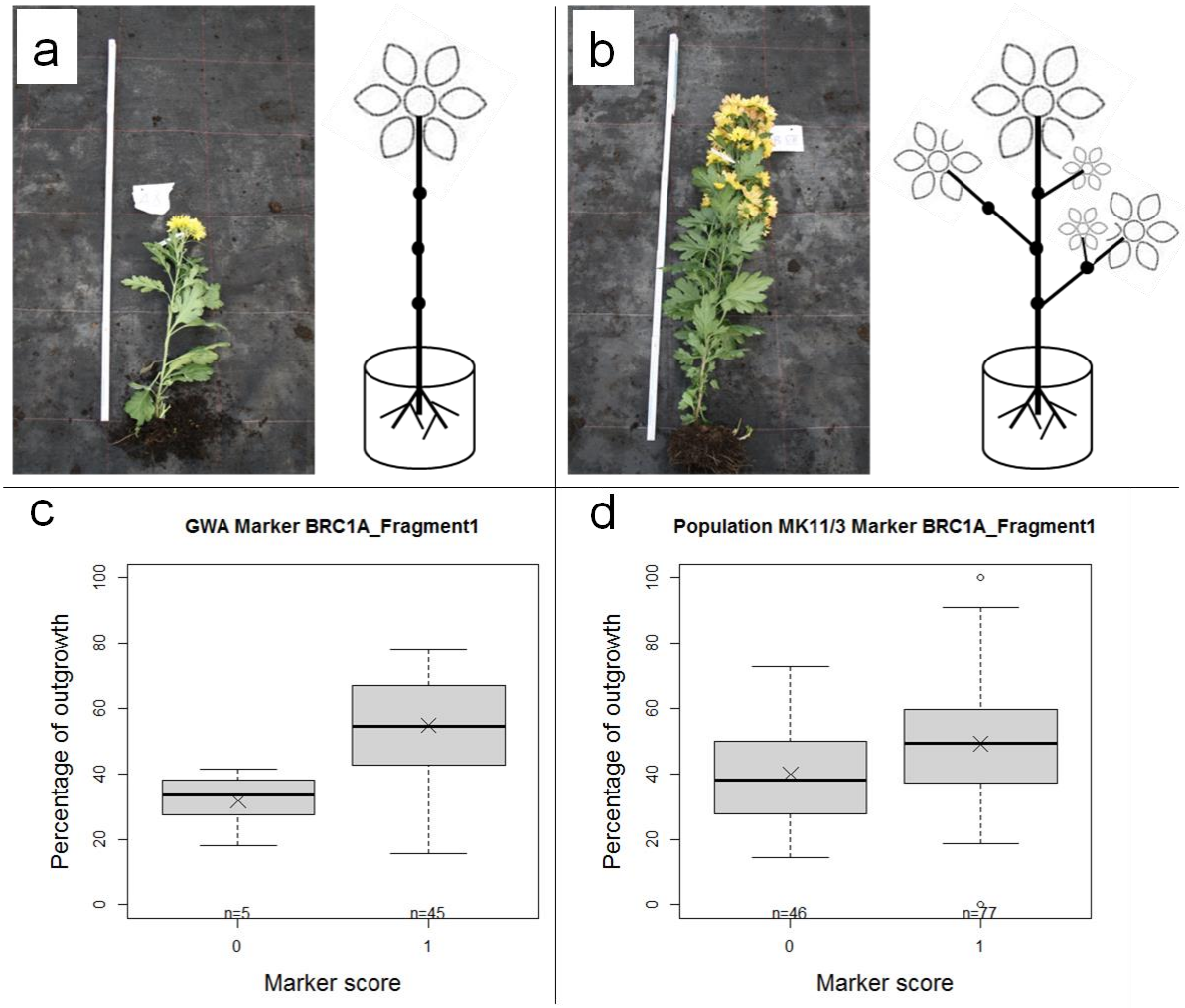

Figure 4.1: a+b) Examples of a weak (a) and a strong (b) branched chrysanthemum genotype. c+d) The phenotypic distribution for shoot branching (percentage of outgrown side shoots from the total number of nodes) according to the presence/absence of a significantly associated marker fragment for the *BRC*1 gene in the GWAS (c) and in the MK11/3 population (d). The boxes represent the 25th and 75th quartiles, the whisker caps indicate the 10th and 90th percentiles, the medians are indicated by the bold line and the mean is represented by x.

As was performed with the AFLP® markers, we analysed the candidate genes in the collection of 81 chrysanthemum genotypes in an association study (MLM of TASSEL with a Q matrix and a kinship matrix as covariates according to the GWAS). In addition, genes significantly associated with shoot branching in the MLM were screened for informative polymorphisms in the segregating F1 MK11/3 population to confirm their role.

All of the analysed candidate genes had significant ($\propto = 0.05$) marker associations for shoot branching (percentage of outgrown side shoots) in the chrysanthemum germplasm. Marker fragments from *CCD*8, *MAX*2 and *BRC*1 only increased the percentage of outgrown side shoots, while other marker fragments from *CCD*7 both increased and decreased this trait (see). Marker-trait associations were confirmed for all of the candidate genes except *MAX2* in the MK11/3 population. All identified marker alleles resulted in more outgrown side shoots per total number of nodes in the segregating population (see Table 4.4).

One polymorphic fragment of the *BRC*1 gene (A_Frag1) was associated with the strongest phenotypic effect in the collection of chrysanthemum varieties. Plants with this fragment had on average 23 % more outgrown side shoots for all nodes than plants without this fragment (Figure 4.1c). In the MK11/3 population, two fragments of the same gene (A_Frag1 and A_Frag10) also resulted in increases of 6 and 9 % (Figure 4.1d) in the percentage of outgrown side shoots. Indeed, in the MK 11/3 population, one polymorphic fragment of *CCD*8 (B_Frag2) produced an even stronger phenotypic effect of 12 %. In general, there was a greater range of phenotypic variance for alleles of the candidate genes in the collection of chrysanthemum varieties than in the MK11/3 population.

Discussion

In our study, we investigated the phenotypic variance and genetic regulation of horticultural traits found in cultivated chrysanthemums using two different strategies. On the one hand, we phenotypically and genotypically characterised a diverse collection of chrysanthemum varieties, including cut, potted and garden varieties. On the other hand, we phenotyped and genotyped a biparental F1 population generated from cut flower varieties. In addition to investigating important horticultural traits such as plant height or inflorescence diameter, we also investigated the shoot branching behaviour of chrysanthemum, as this trait is the driving force shaping plant architecture. Thus, we analysed anonymous AFLP® markers and candidate genes of the *MAX*-pathway and *BRC*1 with known roles in the control of bud outgrowth via the phytohormone strigolactone (SL).

Table 4.4: Summary of the significant associations between markers and shoot branching (percentage of outgrown side shoots from the total number of nodes) for candidate genes in a chrysanthemum germplasm collection (GWAS) and in the MK11/3 population. For the GWAS, markers were identified using the MLM-model of TASSEL; for the MK11/3 population, markers were identified using ANOVA. For both approaches the significance level was $\propto = 0.05$. Additionally, q-values are given based on all markers for one candidate gene used in the analysis.

Gene	GWAS	Effect[1]	p-value	q-value	MK11/3	Effect[1]	p-value	q-value
CCD7	B_Frag3	+9 %	0.003	0.041				
	B_Frag2	+8 %	0.003	0.041				
	B_Frag1	-4 %	0.004	0.041	A_Frag4	+3 %	0.028	0.922
	B_Frag4	-12 %	0.004	0.041	A_Frag11	+4 %	0.040	0.922
	D_Frag2	-7 %	0.011	0.048				
	C_Frag9	+14 %	0.014	0.011				
CCD8	B_Frag2	+9 %	0.043	0.522	B_Frag6	+3 %	0.003	0.082
					B_Frag4	+4 %	0.028	0.318
	B_Frag8	+7 %	0.047	0.522	B_Frag2	+12 %	0.037	0.318
MAX2	B_Frag6	+6 %	0.022	0.065	n. s.			
	B_Frag7	+15 %	0.035	0.074				
BRC1	A_Frag15	+23 %	0.002	0.012	A_Frag1	+9 %	0.004	0.052
					A_Frag10	+6 %	0.041	0.245

Phenotypic characterisation of cultivated chrysanthemums

We phenotyped a collection of 81 chrysanthemum accessions for plant height, internode length, number of nodes, number of formed axillary buds, number of formed side shoots, length of the longest side shoot and inflorescence diameter. All investigated traits varied to a significant extent but were not always normally distributed. For instance, flower size and plant height displayed a bimodal distribution (see supplemental Figure S4.1). This deviation from a normal distribution was most likely caused by the composition of the genotype collection, which was dominated by cut varieties and pot varieties that were considered new varieties based on varying floral traits. In contrast, most of the characterised traits (such as flower size, plant height, number of nodes and number of formed side shoots) were normally distributed in the MK11/3 population. With the exception of the percentage of outgrown side shoots, trait variation was lower in the MK11/3

population than in the collection of varieties. This was expected, as the MK11/3 population is derived from a cross of two varieties, and the allelic composition of these varieties determines the characteristics of the progeny.

Because all phenotypic traits were continuous and close to normally distributed for the MK11/3 population, this finding was consistent with the free combination of most alleles of a segmental allopolyploid (Klie, unpublished observations). Furthermore, quantitative variation is expected for most traits because of the strictly outcrossing hexasomic nature of the chrysanthemum.

Only weak correlations between traits were observed in the collection of chrysanthemum varieties and in the MK11/3 population. As expected, the mostly strongly correlated traits were plant height and the length of the longest side shoot. Surprisingly, there were no correlations between flower size and plant height or number / proportion of outgrown side shoots; such a correlation would be expected, as these traits compete for nutrients and other resources that limit plant growth. However, in other plant species such as *Saxifraga granulata* (Andersson, 1996) or *Eichornia paniculata* (Worley and Barrett, 2000), no trade-off between the number of flowers and the flower size was found. Therefore, it is possible that flower size and number may be influenced by independent genes with only a minor or no effect on the resource status of the plant (Worley and Barrett, 2000).

Because there was a broad spectrum of non-correlated traits, it should be possible to enrich the chrysanthemum gene pool with alleles that are favourable for these traits. Further progress in chrysanthemum breeding could be achieved by crossing genotypes from the extreme ends of the trait distributions. However, chrysanthemums are highly heterozygous and can show self-incompatibility (Drewlow *et al.*, 1973); it can thus be difficult to transfer these alleles to a homozygous state.

Molecular markers associated with phenotypic traits

We applied two different approaches to identify molecular markers linked to phenotypic traits in cultivated chrysanthemums. A genome wide association study (GWAS) identified 15 significant AFLP® markers (after Bonferroni correction) in the collection of 81 chrysanthemum genotypes. The phenotypic effects ranged

from -234 % for the ratio of plant height to the length of the longest side shoot to +31 % for the flower size; however, we were unable to identify markers for all investigated traits. The very large effect of the HAAG_MACCT_135 marker was based on its absence in only three accessions, all of which had relatively larger plant heights than side shoot lengths, resulting in a large ratio of the two values.

The comparison of the ratio between plant height and the length of the longest side shoot is not trivial and results in the strong effect of the abovementioned marker-locus, which is probably very much overestimated by the statistical procedures used. Most of the markers were associated with flower size (seven markers) and produced variations in flower size ranging from 2 to 31 %. In addition, we identified four markers associated with the length of the longest side shoot. Two of these markers increased this trait while the other two decreased it; as a result, it is likely that they are linked to genetic regions that support or inhibit the growth of side shoots.

In our study, we scored 1000 polymorphic AFLP® markers; similar numbers of markers have been studied in GWAS conducted in other non-model plants such as sugarcane (Wei *et al.*, 2006), pine (Quesada *et al.*, 2010) and *Phalaenopsis* (Gawenda *et al.*, 2012). Even though 1000 markers are not sufficient to cover the entire genome of the chrysanthemum, we were able to identify significant marker associations. In the future, more sophisticated association studies with an extremely high marker density will be possible with next-generation sequencing techniques that will allow high-throughput marker analyses in ornamentals. Our study is the first GWAS to provide a preliminary glance into the very complex structure of the chrysanthemum genome and provided a useful way of identifying loci for candidate genes for important horticultural characteristics.

In addition to the GWAS, we scored 327 polymorphic AFLP® markers in the MK11/3 population and used this information to detect QTL with a one-way ANOVA. We corrected the output for false-positive markers by applying the method described by Herrera and Bazaga (2009). This method identified 17 significant markers for important horticultural traits. The phenotypic effects explained by these markers produced variations in mean values ranging from -34 to +27 %. Seven markers were associated with flower size and produced variations

of -13 % to 11 %. Furthermore, association analysis revealed that two markers (HAAT_MACGA _6 and HAAT_MAAGG_23) were each associated with two different traits (percentage of outgrown side shoots and number of formed side shoots and plant height and length of the longest side shoot, respectively). Although these traits were not as highly correlated as in the GWAS, they were nevertheless positively correlated.

The markers significantly associated with important horticultural characteristics in the chrysanthemum, which were detected by the GWAS and QTL analysis, explained phenotypic variations ranging from 2 to 34 %. Only one marker explained with 234% a markedly larger part of the variation. The magnitudes of the marker effects are similar to those reported for inflorescence-related (Zhang *et al.*, 2011a) and flowering traits (Zhang *et al.*, 2011b) in previously published linkage mapping analyses in the chrysanthemum. Because no single marker explained all of the variation in a given trait, it is clear that the analysed traits are controlled by more than one gene and might also be affected by environmental factors. Nevertheless, we were able to detect useful loci for candidate genes for important horticultural traits. However, these yet anonymous loci must be independently confirmed via family-based qualitative and quantitative trait mapping before they can be utilised in marker-assisted breeding. The conversion of an AFLP® marker into a sequence-specific marker is technically difficult, as several different sequences of the same size can be detected by an AFLP® band and sequence-level polymorphisms might therefore be lost.

Candidate gene approach for shoot branching

Recently, SL was characterised as a new phytohormone that inhibits shoot branching (Gomez-Roldan *et al.*, 2008; Umehara *et al.*, 2008). SL was identified after exhaustive mutant screening in *Arabidopsis thaliana* and in the pea, petunia and rice (e. g., recently reviewed by Cheng *et al.*, 2013). These mutant screens led to the identification and biochemical characterisation of several pathway genes, including *CCD*7, *CCD*8 (both carotenoid cleavage dioxygenase) and *MAX*2 (F-box receptor), which are also found in the chrysanthemum (*CCD*7 unpublished; Liang *et al.*, 2010; Dong *et al.*, 2013). *BRC*1, which acts downstream of the SL pathway

and whose expression is enhanced by the phytohormone (Figure 4.2), was characterised in the chrysanthemum as well (Chen *et al.*, 2013).

We screened all four genes for polymorphisms by SSCP analysis in the collection of chrysanthemum genotypes and in the MK11/3 population. Using the MLM approach of the GWAS, we identified marker alleles that were significantly associated with each trait and produced variations in the percentage of outgrowth ranging from -12 to 23 %. When present, most marker alleles resulted in an increased rate of side shoot outgrowth. The *CCD*7 marker alleles produced both increases and decreases in phenotypic traits. This might be because different alleles carry mutations in different regions of the gene and therefore produce different phenotypic effects. In agreement with the results of our study in which one marker allele of *BRC*1 produced the strongest phenotypic effect of all candidate genes, an association study of shoot branching in Arabidopsis also identified *MAX*2 and *CCD*7 as being significantly associated with phenotypic variance (Ehrenreich *et al.*, 2007).

In contrast to the association analyses with AFLP® markers, we confirmed the effect of marker alleles from the candidate genes *CCD*7, *CCD*8 and *BRC*1 on shoot branching by independent analyses in a biparental segregating population. All alleles segregating in the MK11/3 population produced an increase in the percentage of outgrown side shoots (from 6 to 12 %). This finding supports the hypothesis that genes related to the SL pathway and phenotypic differences play a fundamental role in shoot branching in the chrysanthemum (Figure 4.2). This finding allows the impact of these genes to be further evaluated: indeed, the combined effect of all significantly associated markers in the MK11/3 population explains 41 % of the total phenotypic variance. Nevertheless, we are aware that our approach might not have detected all possible marker alleles associated with the candidate genes. Furthermore, it is very likely that shoot branching in the chrysanthemum is influenced by additional loci. Some of these loci may have already been identified in our QTL analysis with AFLP® markers, explaining an additional effect of 24 %. This might be an overestimation, if the AFLP® loci are linked to the candidate genes. The estimation of such a linkage is problematic if small numbers of markers are used in hexaploids.

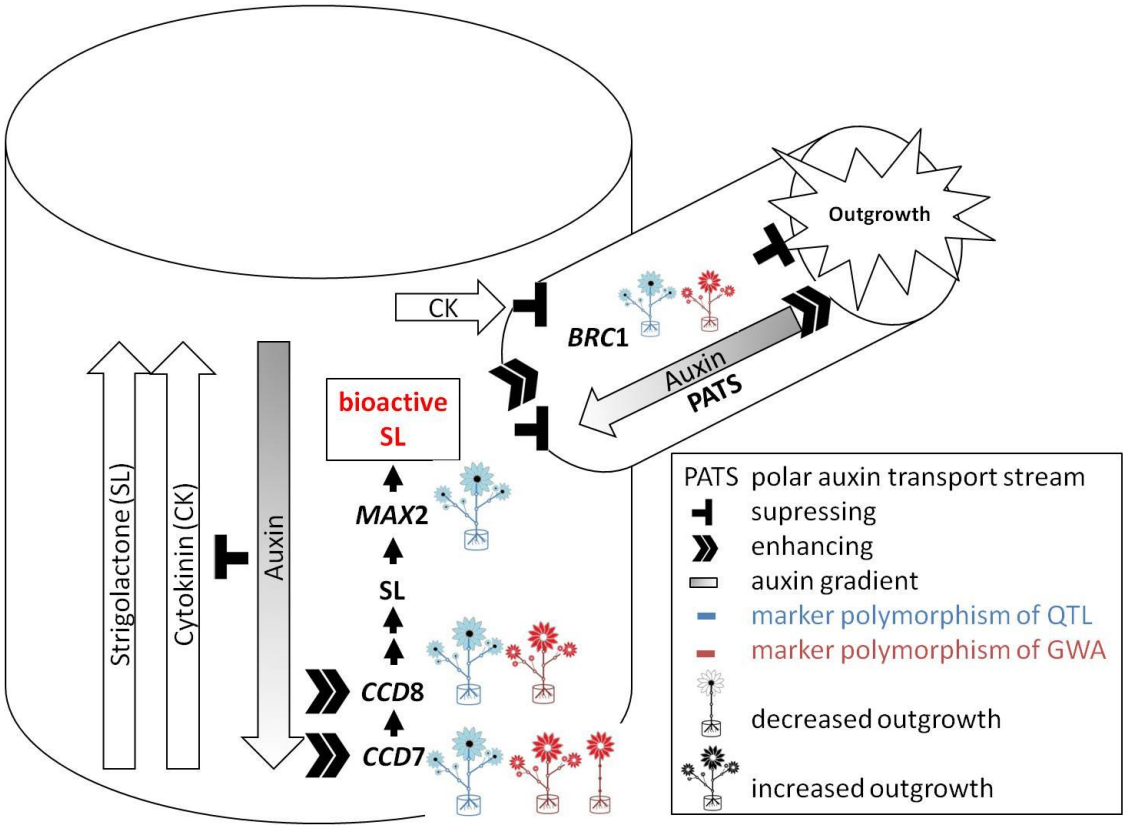

Figure 4.2: A scheme for the effect of marker polymorphisms on shoot branching in the chrysanthemum. The effects on the percentage of outgrown side shoots (increasing or decreasing) and their role in the hormonal regulation of side shoot formation by auxin, cytokinin (CK) and strigolactone (SL) are shown for the candidate genes *CCD*7, *CCD*8, *MAX*2 and *BRC*1. The marker alleles were identified by an association study in a collection of chrysanthemum varieties (red) and a QTL analysis in the F1 MK11/3 population (blue). SL is derived from carotenoid precursors after enzymatic processing by *CCD*7, *CCD*8 and other enzymes and becomes biologically active after recognition by the F-box receptor *MAX*2. Bioactive SL acts both directly and indirectly via auxin on the outgrowth of side shoots. By altering polar auxin transport, SL reduces the capacity of the polar auxin transport stream (PATS) in the main stem, leading to enhanced competition between buds to release their auxin into the stem, thus promoting their outgrowth (Crawford *et al.*, 2010; Shinohara *et al.*, 2013). In contrast, SLs and CK are transported acropetally through the xylem and act directly on the buds to control their outgrowth through the joint regulation of the TCP transcription factor BRC1 (Braun *et al.*, 2012; Dun *et al.*, 2013).

Our findings differ from those found for *Helianthus annuus*, another member of the *Asteraceae* family. Rojas-Barros *et al.* (2008) identified one single dominant apical branching gene in the cultivated sunflower. The gene was fine-mapped close to TRAP markers derived from the candidate genes *CCD*7 and *CCD*8. Currently, the branching locus of *H. annuus* has not been characterised and it is not clear whether *CCD*7 and/or *CCD*8 might also influence apical branching, as the effects of these genes are masked by the dominant effect of the dominant single major locus. Our

results suggest a different possibly polygenetic mechanism for the genetic control of branching in the chrysanthemum. Furthermore, the strigolactone pathway does not completely explain the phenotypic variation in shoot branching found in our study.

Conclusions

All horticultural traits analysed in this study varied quantitatively independent of the type of population used (collection of cultivars or biparental F1 population MK11/3). It is therefore very likely that these characteristics are inherited polygenetically. This hypothesis was supported by the identification of marker alleles that were associated with several of the traits but did not explain the phenotypic variance completely. A detailed analysis of candidate genes for shoot branching in the chrysanthemum further confirms this hypothesis. All candidate genes of the SL pathway and *BRC*1 had marker alleles that were significantly associated with shoot branching in both types of populations, producing variations of up to 23 % for a single marker; the only exception was *MAX*2 in the biparental MK11/3 population. In addition, anonymous AFLP® markers were found to be associated with this trait and produced 7 to 16 % of the phenotypic variance.

Because of the complex genetics of the chrysanthemum, the results of this study did not allow us to select for single preferred alleles. Nevertheless, the identified marker-trait effects could be validated by targeted crossing of genotypes carrying these alleles. If these alleles show a significant effect that is beneficial to the breeding process and end up in the progeny, they might be useful for breeding new varieties in the future.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MK did the experimental work and participated in the writing of the manuscript. ML and TD planned and designed the project and participated in the writing of the manuscript. All authors have read and approved the final manuscript.

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5 Identification of a *CCD*7/*MAX*3 orthologue and the role of the strigolactone pathway for shoot branching in chrysanthemum

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Abstract

Chrysanthemums (*Chrysanthemum indicum* hybrid) are important morphologically highly diverse ornamental plants used as cut flowers and potted plants. In commercial chrysanthemum cultivation, shoot architecture is managed by manual disbudding which is time consuming, costly and favours infection by fungi and bacteria. Therefore, novel genetic approaches to control shoot branching would be advantageous. Here, we used different weak and strong branched genotypes of the MK11/3 population to investigate the physiology of shoot branching after decapitation and under different concentrations of the strigolactone (SL) analogue CISA-1. In addition, we characterised the yet unknown *CCD*7 gene of the chrysanthemum and analysed the expression of SL pathway genes in the aforementioned genotypes. Our results indicate that shoot branching of *in vitro* plantlets of chrysanthemum is inhibited by CISA-1 and induced by decapitation. Decapitation is a severe stress factor and induces dramatic changes on the expression of SL pathway genes in accordance with the enhanced outgrowth of side shoots. By the characterisation of a putative *CCD*7 orthologue we deciphered another step in the SL pathway of chrysanthemums. Our study highlights the importance of SL in the regulation of shoot branching in the chrysanthemum; though, other yet unknown factors are also likely involved.

Key words

bud outgrowth, CISA-1, decapitation, *MAX* pathway, qPCR, strigolactone analogue

Introduction

Shoot branching is shaping the above ground habit of a plant and it is therefore crucial for the aesthetic value of ornamental crops. Chrysanthemums (*Chrysanthemum indicum* hybrid) are important ornamental plants with diverse shoot architecture due to their use as cut flowers and potted plants. In the cultivation of chrysanthemum, pinching, bud picking and disbudding need to be performed manually to improve shoot architecture, accounting for approximately one-third of the total cultivation costs (Dong *et al.*, 2013). Therefore, novel approaches are needed to control shoot branching in chrysanthemum.

Shoot branching in plants is a complex trait. It is affected by the genetic background, gene expression, phytohormones, the environment, and the interaction of these components. Two different processes mainly influence it: the initiation of axillary meristems (AM) and their outgrowth. Both processes have already been investigated in chrysanthemums. Yang *et al.* (2005) characterised the *LATERAL SUPRESSOR-LIKE* (*DgLsl*) gene from chrysanthemum and it was used to generate non-branching chrysanthemum lines by a transgenic approach (Han *et al.*, 2007). These plants showed less branching because their capacity to initiate AMs was reduced (Han *et al.*, 2007). Recently, strigolactone (SL) was characterised as a new phytohormone, which controls the outgrowth of side shoots and thus the breaking of bud dormancy (Gomez-Roldan *et al.*, 2008; Umehara *et al.*, 2008). Several genes of the SL pathway, such as *CCD*8 (SL synthesis, Liang *et al.*, 2010) and *MAX*2 (SL signalling, Dong *et al.*, 2013), were identified. Additionally, Chen *et al.* (2013) characterised the orthologue of the TCP transcription factor BRC1 of chrysanthemum that is involved in the SL mediated response and directly inhibits the outgrowth of side shoots (Crawford *et al.*, 2010; Shinohara *et al.*, 2013). All SL pathway related genes have not been characterised yet and this study aims to identify the *CCD*7 gene and to improve our knowledge about shoot branching in the chrysanthemum.

Therefore, we used the sequence information of publicly available *CCD*7 genes to amplify parts of the putative *CCD*7 orthologue of chrysanthemum and 5' -and 3'

Rapid Amplification of cDNA Ends (RACE) PCR to recover the complete coding sequence (CDS). Additionally, we analysed the shoot branching phenotype of selected genotypes of the MK11/3 population during *in vitro* cultivation and their branching patterns under decapitation and treatment with the synthetic SL analogue CISA-1 (Rasmussen *et al.*, 2013). Furthermore, we studied the gene expression of candidate genes (*CCD*7, *CCD*8, *MAX*2 and *BRC*1) with known function in the regulation of bud outgrowth. Thus, our study provides insight into the regulation of shoot branching by SL in chrysanthemum.

Materials and Methods

Plant material

Plantlets of the *Chrysanthemum indicum* hybrid cultivar "Relinda" (VZR) were cultivated in 7.5-L pots with a fertilised substrate ("Einheitserde T") in a greenhouse under a 16 h light/8 h dark cycle at a constant temperature of 22 °C. During the vegetative growth phase shoot tips were removed for RNA extraction.

We established a segregating biparental F1 population (MK11/3) of 160 individuals by crossing the female parent *C. indicum* hybrid 'Kitam' (541) with the paternal parent VZR. One clone of each F1 genotype was cultivated in three independent randomised blocks as described in Klie *et al.*, unpublished. The F1 plants were grown in a greenhouse under a 16 h light/8 h dark cycle at a constant temperature of 22 °C from week 34 until week 38. Subsequently, the cycle was changed to 8 h light/16 h dark to induce flowering. Data on the shoot branching traits (number of nodes and number of formed side shoots) were collected for all three clones of the 160 genotypes after senescence of the flowers. Based on their branching phenotype the siblings MK11/3-19, -21, -66 and -104 were selected for further experiments.

In-vitro-culture

A sterile *in vitro* culture was established with plantlets of the siblings MK11/3-19, -21, -66 and -104 by collaborators of the company Hubert Brandkamp. The plantlets were cultivated and propagated in Murashige and Skoog (MS) media (Murashige and Skoog, 1962) under a 16 h light/8 h dark cycle at a constant

temperature of 22 °C. These four genotypes were used in different experiments to analyse shoot branching in the chrysanthemum.

In a decapitation assay three to five plantlets of 6 to 8 cm height without roots of each genotype were transferred to MS media in sterile 0.5 L plastic containers. One half of the plantlets were decapitated above the first fully opened leave. Both intact and decapitated plantlets were transferred to plastic containers with MS media containing 0, 0.5, 2.5 and 5 µM of the SL analogue CISA-1 (dissolved in 1 mL acetone; Rasmussen *et al.*, 2013). For each of the genotypes and treatments three to four plastic containers with three to five plantlets were used. The individual treatments were repeated at least twice, except for the treatment of the genotypes MK11/3-19 and -66 with 0.5 and 2.5 µM CISA-1 and the treatment of the genotypes MK11/3-21 and -104 with 0 and 5 µM CISA-1. Data on the shoot branching traits (number of nodes and number of formed side shoots) were collected after four to six weeks of cultivation.

In vitro plantlets with four fully opened leaves of the genotypes MK11/3-19 and -66 were used for an expression analysis. Three plantlets per genotype were transferred to 0.5 L plastic containers with MS media. For each genotype three to four containers were used for each decapitation experiment. The decapitation experiment was repeated two times. For RNA isolation 30 mg of roots and of stem segments carrying a node were sampled before and 12 h as well as 7 d after decapitation above the first fully opened leave.

RNA isolation

The sampled tissues were frozen in liquid nitrogen and ground for 2.5 min using a bead mill at 26 s^{-1}. Total RNA was extracted using the Quick-RNA™ MiniPrep Kit (Zymo Research, Freiburg, D) according to the manufacturer's instructions. The remaining DNA was removed by DNase treatment directly on the column during the isolation. The RNA concentration was assessed spectrophotometrically at 260 nm and was checked for purity by determining the OD 260 nm/280 nm and the OD 260 nm/230 nm ratios. The RNA quality was assessed by gel electrophoresis.

cDNA synthesis

For each sample, 300 ng of total RNA was reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Austin, USA) with random hexamer primers according to the manufacturer's instructions. The cDNAs were diluted 1:5 with nuclease-free water prior to the qPCR analyses.

Isolation of the putative chrysanthemum *CCD*7 gene

Primers were designed for highly conserved regions of the *CCD*7 gene based on the alignment of publicly available *CCD*7 cDNA sequences of *Arabidopsis thaliana*, *Artemisia annua* L. and tomato (*Solanum lycopersicum*). These primers (forward primer 5'-CCCTCTAGATGGTCATGG-3' and reverse primer 5'-GCATTGCATGACATCATAAG-3') were used to amplify a fragment of the chrysanthemum *CCD*7 gene in a 50 µL reaction volume composed of 1 x Hi-Fi Buffer, 0.2 mM dNTPs, 0.5 µM primers, 1 U Velocity Taq polymerase (Bioline GmbH, Luckenwalde, D) and 1 µL of template cDNA. Conditions for the PCR amplification were as follows: 98 °C for 2 min, 35 cycles at 98 °C (30 s)/60 °C (30 s)/72 °C (30 s) followed by a final extension at 72 °C for 4 min. The PCR fragment was extracted using the GeneJET Gel Extraction Kit (Thermo Fisher Scientific Germany GmbH, Schwerte, D) after agarose gel electrophoresis and was subsequently cloned into *Escherichia coli* K12/DH10B using the pJet1.2 cloning vector (Thermo Fisher Scientific Germany GmbH, Schwerte, D) according to the manufacturer's instructions. Plasmid isolations were carried out using Nucleospin® Plasmid kits (Macherey-Nagel GmbH & Co. KG, Dueren, D) according to the manufacturer's instructions. Plasmid DNA carrying the desired fragments were sequenced by a commercial sequencing company.

For 3' RACE, cDNA was synthesized with a tailed oligo-dT adapter primer (AP; Life Technologies GmbH, Darmstadt, D). The 3' RACE PCR fragments were amplified by PCR using an abridged universal amplification primer (AUAP; Life Technologies GmbH, Darmstadt, D) and a specific primer (5'-AGCAAGATCTAACAAGTCCACACCAC-3'). For 5' RACE, the FirstChoice® RLM-RACE Kit -tailed cDNA (Life Technologies GmbH, Darmstadt, D) was used with the specific primer 5'-ATGCAGGCTAAAGCATTCAATT-3' according to the manufacturer's instructions using Phusion High-Fidelity DNA polymerase (Biozym

Scientific GmbH, Oldendorf, D) for PCR. Amplified fragments were cloned and sequenced as described before.

Based on the sequence of the 5' - and 3' RACE, a forward primer (5'-ATGCAGGCTAAAGCATTCAATT-3') from the 5' end of the coding sequence (CDS) and a reverse primer (5'-CCCAAAAGCCATGAAATCCAA-3') from the 3' end of the CDS of the *CCD*7 gene were designed and used to amplify the complete CDS from cDNA. The complete CDS of the *CCD*7 gene was then cloned and sequenced as described before.

Quantitative PCR

The genotypes MK11/3-19 and -66 were selected for expression analysis due to their difference in shoot branching. We used six biological samples of both genotypes for each treatment. Such a sample was examined in four technical replicates, which comprised of two independent PCR runs and in each run every amplification reaction was repeated once.

The amplification reactions were performed on transparent 0.1 mL 96-well plates (Sarstedt AG & Co., Nümbrecht, D) using SYBR Green detection chemistry and run on the StepOnePlus™ System (Applied Biosystems, Austin, USA). The reactions were prepared in a total volume of 10 µL containing 2 µL of template, 1 µL of each amplification primer [0.25 nM], 5 µL of 2x SensiMix SYBR (Bioline GmbH, Luckenwalde, D) and 1 µL of nuclease-free water. The water-only controls included 3 µL of nuclease-free water instead of a cDNA template and were run for each primer pair on each plate. The cycling conditions were set as follows: initial denaturation for 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 15 s at 60 °C and 15 s at 60 °C. The amplification specificity for each primer pair was tested by a melting curve analysis ranging from 60 to 90 °C with temperature steps of 0.5 °C. The different primer pairs and targeted genes are listed in Table 5.1. The PCR products were further analysed on 3 % agarose gels. The StepOne™ Software (Applied Biosystems, Austin, USA) was used to perform the baseline correction and for automatic determination of the quantification cycle (Cq). The data were exported to MS Excel for further statistical analysis.

Table 5.1: Primer sequences and amplicon characteristics for each of the used genes in the qPCR assay.

Gene	Accession		Primer Pairs	Product Size
CCD7	unpublished	F	CGTTGGATACCCTTGGAAAGT	94 bp
		R	CCACCAACCTTCTCATCACAC	
CCD8	Liang *et al.*, 2010	F	GGTGAAGGCTACGCGCTAATA	80 bp
		R	TATGGAAGCCCATAAGGGAAC	
MAX2	JX556222	F	GCACATACTGCACCATC	142 bp
		R	GTAACGACAAACTCCTCTGG	
BRC1	JX870411	F	TGCAGCATCAGTTCAGTGACT	106 bp
		R	TGCTCATGCCTTCCCTGTTAG	
GAPDH	Gu *et al.*, 2011	F	CTGCTTCTTTCAACATCATTCC	170 bp
		R	CTGCTCATAGGTAGCCTTCTTC	
UBC	Gu *et al.*, 2011	F	CATCTACTCGTCAATCAGGGTT	185 bp
		R	GTATGGGCTATCGGAAGGTC	

Data analysis

The phenotypic data were analysed using the packages mvtnorm, multcomp and gplots of the R software version 2.15.2 (R Core Team, 2012). To measure the branching propensity of the plants, we estimated the ratio of formed side shoots to the number of nodes. To achieve a normal distribution for these ratios we logarithmically transformed these ratios. For multiple testing a Tukey test or a Dunnett test with $\alpha = 0.05$ was used.

The alignment of the publicly available *CCD*7 sequences was performed using the ClustalW algorithm for multiple alignments of the BioEdit software version 7.2.0 (Hall, 1999). The sequence information of the cloned fragments of the

putative *CCD*7 gene was processed and the CDS was also translated into its amino acid (AA) sequence using the BioEdit software version 7.2.0. The translated AA sequence and sequences of other publicly available carotenoid cleavage dioxygenases (CCDs) were aligned using the PRANK algorithm of the GUIDANCE homepage (http://guidance.tau.ac.il/) for subsequent phylogenetic analyses. Different amino acid substitution models were tested for the alignment in Mega5 (Tamura *et al.*, 2011) and the Whelan And Goldman model (WAG) with evolutionary rates following a discrete gamma distribution (+G) was revealed as the most likely model. The alignment was used to generate a phylogenetic tree in Mega5 using the Maximum Likelihood method based on the WAG+G model. Positions containing gaps and missing data were partially deleted. To evaluate the robustness of the dendrogram, a bootstrap analysis with 1000 replicates was conducted.

To evaluate the gene expression data the Cq values of the different qPCR runs were summarized and formatted for its further use with MS Excel. The data was analysed using the qpcrmix package (Gerhard *et al.*, 2013) of the R software version 2.15.2.

Results

Phenotypic characterisation of the selected genotypes of the MK11/3 population

The MK11/3 population was phenotyped in depth for flower size, plant height, number of nodes, number of formed side shoots, and length of the longest side shoot in the greenhouse (Klie *et al.*, unpublished). To measure the branching propensity of the plants, we estimated the ratio of formed side shoots to the number of nodes. Based on these data the genotypes MK11/3-19 and -21 (18.3 % and 20.6 %, respectively) were weakly branched, whereas the genotypes MK11/3-66 and -104 (82.3 % and 78.9 %, respectively) were strongly branched.

To further characterise this trait, plantlets of these genotypes were transferred to the *in vitro* culture. Shoot branching of intact and decapitated plantlets was recorded. There was a strong effect of the decapitation on the shoot branching of

all four genotypes, as the number of outgrown side shoots increased in comparison to intact plantlets (Figure 5.1).

The difference in shoot branching was only significant in a Tukey test ($\alpha = 0.05$) between the genotypes MK11/3-19 and -66 and between -21 and -66 for intact plantlets (Figure 5.1a), while under greenhouse conditions MK11/3-19 and -21 were significantly less branched (Tukey test, $\alpha = 0.05$; data not shown) than the genotypes MK11/3-66 and -104. For decapitated plantlets, only the genotypes MK11/3-21 and -104 were significantly different (Figure 5.1b). Indeed, we are aware that the tests are affected by the different number of observations for each genotype due to a different number of experimental repetitions.

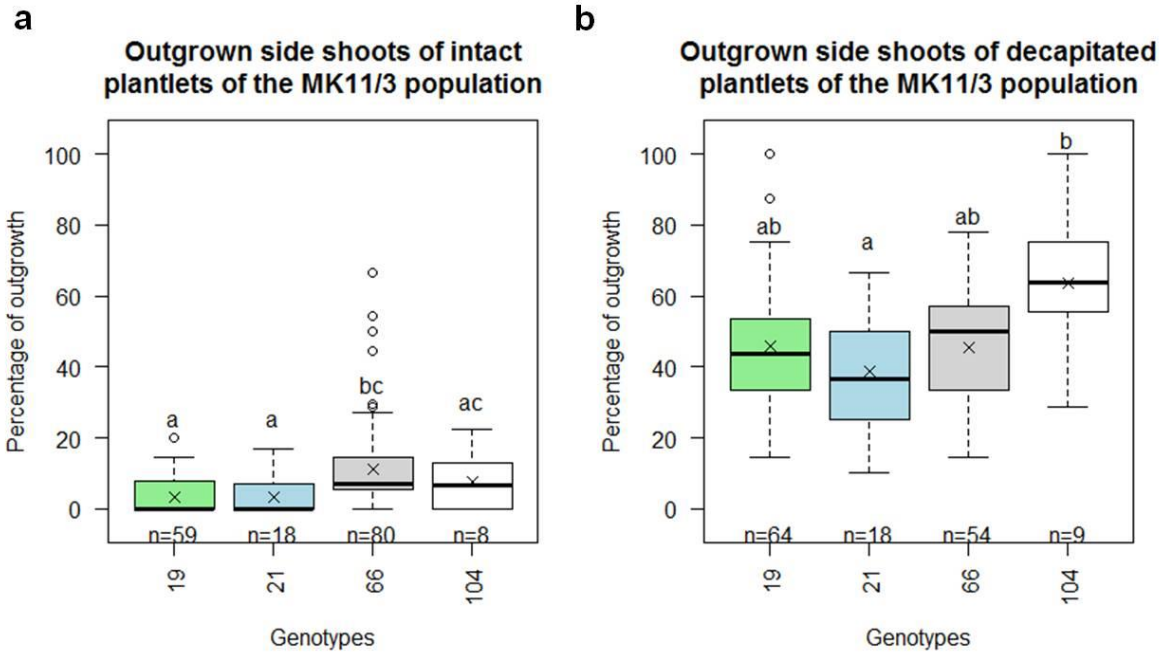

Figure 5.1: The phenotypic distribution for shoot branching (percentage of outgrown side shoots from the total number of nodes) for selected genotypes of the MK11/3 population. a) intact *in vitro* plantlets; b) decapitated *in vitro* plantlets. The boxes represent the 25th and 75th quartiles, the whisker caps indicate the 10th and 90th percentiles, the medians are indicated by the bold line and the mean is represented by x, the different colours indicate the different genotypes, the different letter indicate significant differences of the Tukey test ($\alpha = 0.05$).

The inhibition of chrysanthemum buds by the SL analogue CISA-1

Recently, strigolactone (SL) was characterised as a phytohormone that inhibits the outgrowth of axillary buds. The physiological effects of SL have been studied in

several plant species using SL analoga which are much easier to generate as genuine SL (Gomez-Roldan *et al.*, 2008; Braun *et al.*, 2012; Hamiaux *et al.*, 2012; Rasmussen *et al.*, 2013). Therefore, we treated intact and decapitated *in vitro* plantlets of the four different selected genotypes of the MK11/3 population with the synthetic SL analogue CISA-1 and monitored their shoot branching reaction.

The application of 5 µM CISA-1 significantly reduced (t-Test, $\alpha = 0.05$) the bud outgrowth of intact as well as decapitated plantlets of all genotypes in comparison with control (0 µM CISA-1) plantlets (data not shown). However, each individual genotype reacted differently to the treatment with the phytohormone. For intact plantlets, only MK11/3-66 showed significantly less (t-Test, $\alpha = 0.05$) shoot branching with 5 µM CISA-1 (Figure 5.2a). While, shoot branching is significantly decreased (t-Test, $\alpha = 0.05$) for the genotypes MK11/3-19 and -66 with 5 µM CISA-1 after decapitation (Figure 5.2b). Indeed, the number of analysed plants

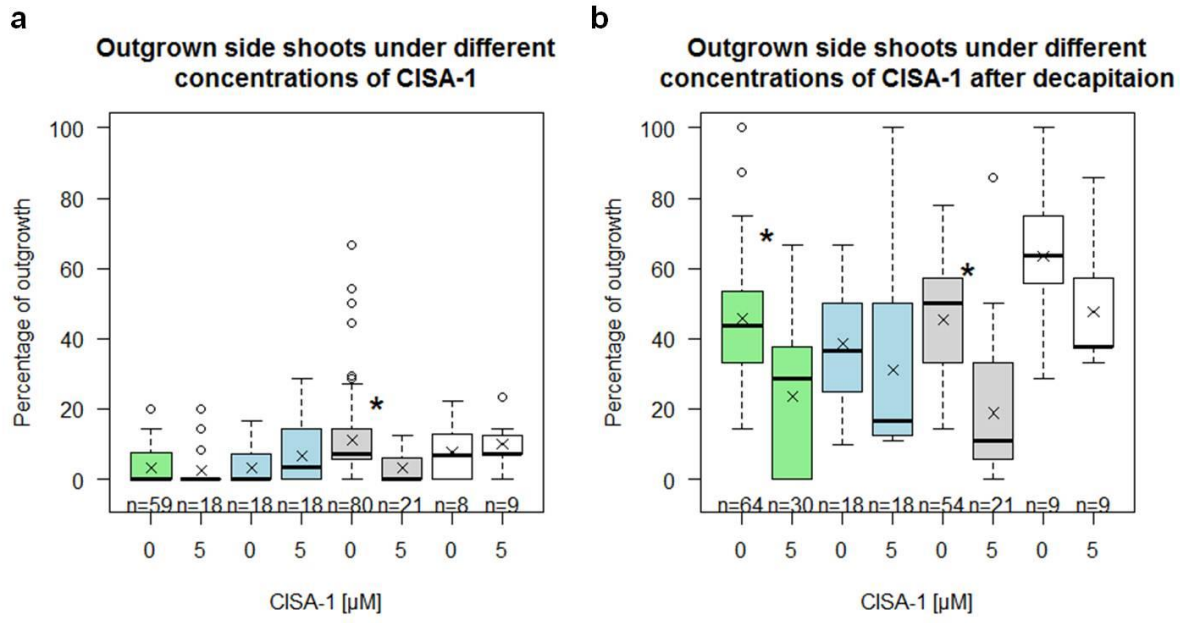

Figure 5.2: The phenotypic distribution for shoot branching (percentage of outgrown side shoots from the total number of nodes) for selected genotypes of the MK11/3 population after hormone treatments with 5 µM strigolactone analogue CISA-1. a) intact *in vitro* plantlets; b) decapitated *in vitro* plantlets. The boxes represent the 25th and 75th quartiles, the whisker caps indicate the 10th and 90th percentiles, the medians are indicated by the bold line and the mean is represented by x, the different colours indicate the different genotypes (19 = green, 21 = blue, 66 = grey and 104 = white), the asterisk represents a significant difference (t-Test, $\alpha = 0.05$) of the hormone treatment for one genotype.

differs between the individual genotypes because of the availability of proper plant material and a small amount of available CISA-1.

An additional experiment with the genotypes MK11/3-19 and -66 was conducted with other concentrations of CISA-1 with intact and decapitated plantlets. These genotypes were chosen because intact *in vitro* plantlets of MK11/3-19 (mean of 3.3 % of outgrown side shoots) showed significantly (t-Test, $p = 1.1 \times E - 06$) less shoot branching than MK11/3-66 (mean of 11 %). Using intact plantlets of MK11/3-19 treated with CISA-1 concentrations of 0.5 µM, 2.5 µM and 5 µM in comparison to the CISA-1 zero check, no significant effect on shoot branching was detected in a Dunnett test ($\alpha = 0.05$, Figure 5.3). Whereas, the same testing for the genotype MK11/3-66 revealed a significant difference for the treatment with 0.5 µM, 2.5 µM and 5 CISA-1 in comparison to the CISA-1 zero check (Figure 5.3).

However, the two chrysanthemum genotypes behaved in a different way after decapitation. The genotypes did not differ in their shoot branching phenotype for none of the tested concentrations of CISA-1. In both genotypes the highest dosage of CISA-1 (5 µM) significantly inhibited (Dunnett test, $\alpha = 0.05$) shoot branching of plantlets in comparison with the control group (0 µM; Figure 5.3).

Isolation and characterisation of the putative *CCD7* gene of chrysanthemum

To get further information on the regulation of shoot branching in the chrysanthemum via SL, its putative *MAX3*/*CCD7* orthologue was isolated. Comparison of publicly available *CCD7* sequences of *A. thaliana*, *A. annua* L. and tomato enabled the design of primers based on the most conserved domains of the gene sequences. An approximately 600 bp fragment with strong sequence similarity to the CCD7 family was successfully amplified. The remaining 5' -and 3' region of the coding sequence (CDS) of the chrysanthemum *CCD7* gene was recovered by 5' and 3' RACE PCR. It covered 1851 bp encoding a predicted protein of 616 amino acids (AA).

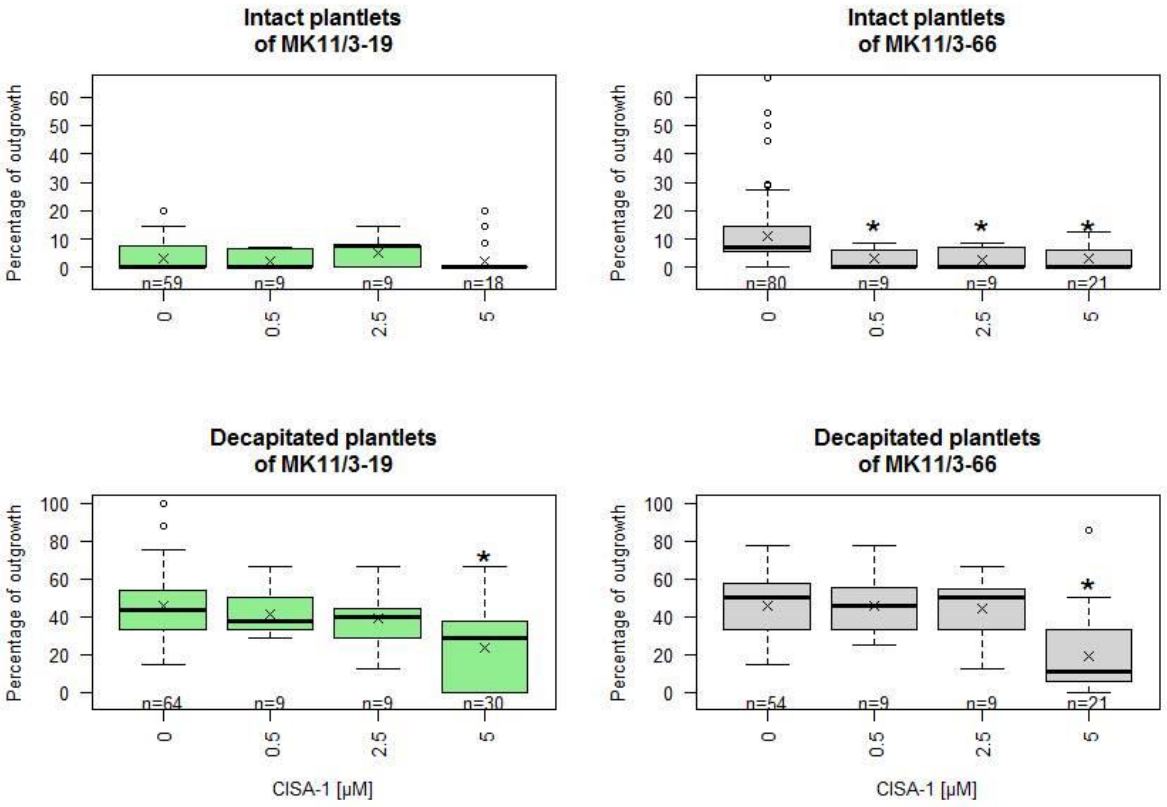

Figure 5.3: The phenotypic distribution for shoot branching (percentage of outgrown side shoots from the total number of nodes) for intact and decapitated plantlets of the genotypes MK11/3-19 and -66 after hormone treatments with the strigolactone analogue CISA-1. The boxes represent the 25th and 75th quartiles, the whisker caps indicate the 10th and 90th percentiles, the medians are indicated by the bold line and the mean is represented by x, the different colours indicate the different genotypes (19 = green, 66 = grey), the asterisk represents a significant difference (Dunnett Test, $\alpha = 0.05$) between the control (0 µM CISA-1) and the applied concentrations of CISA-1 (0.5 µM, 2.5 µM and 5 µM).

AA sequence comparisons between the identified putative gene and its orthologues from *A. thaliana*, *A. annua* L. and tomato showed that the predicted CCD7 sequence is 63% identical to AtMAX3, 94% identical to AaCCD7 and 68% identical to SlCCD7.

To further investigate the relationship between the predicted CCD7 protein, a maximum likelihood tree of characterised CCD family proteins (CCD1, 2, 4, 7 and 8) was constructed from a taxonomically diverse set of plant species (Figure 5.4). In line with their function in the SL pathway, the CCD7 and CCD8 proteins are placed more close to each other than to any of the other CCD family proteins (CCD1, 2 and

4). Furthermore, the chrysanthemum CCD7 forms a well-supported group with other CCD7 proteins of species of the *Asterids* clade (*A. annua*, *Act. chinensis*, *S. lycopersicum* and *P. hybrida*) in accordance with their taxonomic relationship.

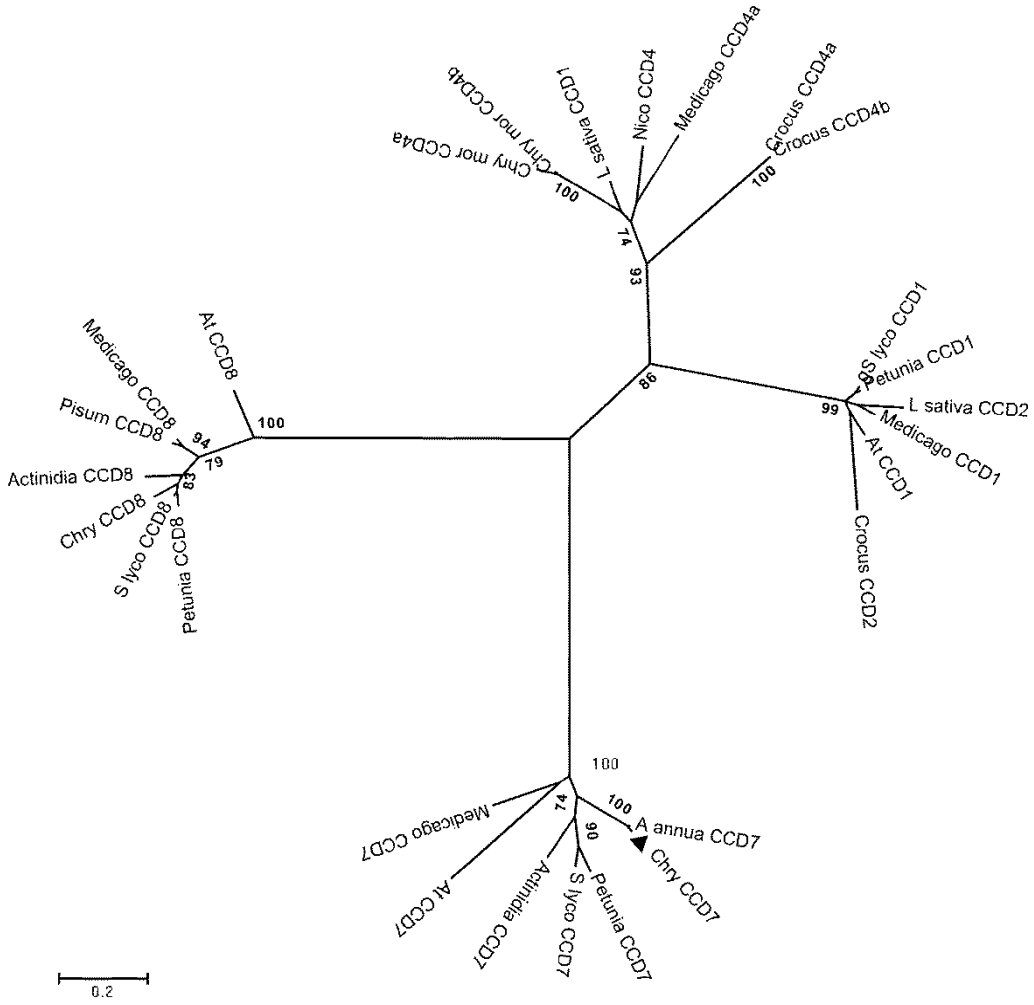

Figure 5.4: Molecular phylogenetic analysis of the putative chrysanthemum CCD7. A maximum likelihood phylogenetic tree of the amino acid sequences of the chrysanthemum CCD7 and characterised CCD orthologues of different plant species is shown. Numbers at the branch nodes indicate bootstrap values from 1000 replications. Only bootstrap values above 70 % are displayed. The branch length is proportional to the sequence distance. The chrysanthemum CCD7 is marked with a filled triangle.

Expression analysis of *CCD7* and other SL pathway genes in chrysanthemum

The genotypes MK11/3-19 and -66 were selected due to their difference in shoot branching. Plantlets of these genotypes were cultivated *in vitro* and RNA was extracted from roots and stem segments with nodes before (T_0), 12 h (T_1) and 7 d (T_2) after decapitation for gene expression analysis via qPCR. The analyses were

conducted with *MAX*2 (Dong *et al.*, 2013), *CCD*8 (Liang *et al.*, 2010) *BRC*1 (Chen *et al.*, 2013) and the putative *CCD*7 gene of chrysanthemum. The *GAPDH* gene (Gu *et al.*, 2011) and the *UBC* gene (Gu *et al.*, 2011) were used as reference genes according to the results of a pre-test (data not shown).

For the comparison of the different tissues of both genotypes, *CCD*7 was significantly less expressed (about 90 %) in the stem as compared to roots. *CCD*8 transcripts were more abundant in the stem fragments with nodes than in roots (Table 5.2 or supplemental Figure S5.1), although the comparison between stem and roots of the genotype MK11/3-66 was not significant. In both genotypes, the *MAX*2 gene and especially the *BRC*1 gene (highly significant) were stronger expressed in stems with nodes than in roots (Table 5.2 or supplemental Figure S5.1).

Table 5.2: Expression analysis of the SL pathway genes (*CCD*7, *CCD*8 and *MAX*2) and *BRC*1 in different tissues (stem segments with nodes and roots) of the chrysanthemum genotypes MK11/3-19 and -66. The expression is shown as relative quantities and was estimated across different time points (before, 12 h and 7 d after decapitation) using the qpcrmix package (Gerhard *et al.*, 2013) with the R software version 2.15.2. The p-values were also estimated by the package and were compared to $\alpha = 0.05$ for significance (*).

Gene	Comparison	$RQ = 2^{\Delta\Delta Cq}$	Mean $\Delta\Delta Cq$	$\Delta\Delta Cq$ std. error	p-value ($\alpha = 0.05$)
CCD7	MK11/3-19 stem with nodes vs. roots	0.08	-3.59	0.73	4.70E-06*
	MK11/3-66 stem with nodes vs. roots	0.09	-3.42	0.61	4.25E-07*
CCD8	MK11/3-19 stem with nodes vs. roots	5.01	2.33	0.98	2.03E-02*
	MK11/3-66 stem with nodes vs. roots	1.72	0.78	1.21	5.21E-01
MAX2	MK11/3-19 stem with nodes vs. roots	2.08	1.05	0.72	1.48E-01
	MK11/3-66 stem with nodes vs. roots	1.67	0.74	0.63	2.44E-01
BRC1	MK11/3-19 stem with nodes vs. roots	11.07	3.47	0.80	4.83E-05*
	MK11/3-66 stem with nodes vs. roots	16.85	4.07	0.54	1.20E-10*

As mentioned before intact plantlets of the genotypes MK11/3-19 and -66 differed in their shoot branching phenotype. Therefore, we compared the expression of the candidate genes between these genotypes before decapitation. Applying the strict criteria of the qpcmix package for statistically significant differences, the expression of none of the analysed genes was significantly

different between MK11/3-19 and -66 (Table 5.3 or see supplemental Figure S5.1). However, there was a tendency that transcripts of all genes were more abundant in the strong branched genotype MK11/3-66, except for *MAX*2 in stems.

Table 5.3: Expression analysis of the SL pathway genes (*CCD*7, *CCD*8 and *MAX*2) and *BRC*1 in different tissues (stem segments with nodes and roots) of the chrysanthemum genotypes MK11/3-19 and -66 before decapitation. The expression is shown as relative quantities and was estimated between the genotypes using the qpcrmix package (Gerhard *et al.*, 2013) with the R software version 2.15.2. The p-values were also estimated by the package and were compared to $\alpha = 0.05$ for significance (*).

Gene	Comparison	Tissue	$RQ = 2^{\Delta\Delta Cq}$	Mean ΔΔCq	ΔΔCq std. error	p-value ($\alpha = 0.05$)
CCD7	MK11/3-19 vs. -66	stem with nodes	0.89	-0.17	0.99	0.86
	MK11/3-19 vs. -66	roots	0.64	-0.65	1.24	0.60
CCD8	MK11/3-19 vs. -66	stem with nodes	0.42	-1.25	1.51	0.41
	MK11/3-19 vs. -66	roots	0.13	-2.91	2.18	0.19
MAX2	MK11/3-19 vs. -66	stem with nodes	1.17	0.23	0.93	0.81
	MK11/3-19 vs. -66	roots	0.49	-1.03	1.30	0.43
BRC1	MK11/3-19 vs. -66	stem with nodes	0.70	-0.51	0.79	0.52
	MK11/3-19 vs. -66	roots	0.60	-0.74	1.37	0.59

As decapitation breaks down the apical dominance of the shoot apical meristem leading to bud outgrowth, we investigated the expression of the candidate genes in the stem before and after decapitation. Both of the genotypes reacted on the decapitation stress by reducing the expression of all of the analysed genes at T_1 - 12 h after decapitation (Table 5.4 or see supplemental Figure S5.1). After 7 d at T_2 the expression of all of the genes increased again, except for the *CCD*8 gene of genotype MK11/3-66 (Table 5.4). However, none of the differences in expression was statistically significant, except of one for *CCD*7.

Table 5.4: Expression analysis of the SL pathway genes (CCD7, CCD8 and MAX2) and BRC1 in stem segments with nodes of the chrysanthemum genotypes MK11/3-19 and -66 before (T0), 12 h (T1) and 7 d (T2) after decapitation. The expression is shown as relative quantities and was estimated between the time points using the qpcrmix package (Gerhard et al., 2013) with the R software version 2.15.2. The p-values were estimated by the package and were compared to $\alpha = 0.05$ for significance (*).

Gene	Genotype	Comparison	$RQ = 2^{\Delta\Delta Cq}$	Mean ΔΔCq	ΔΔCq std. error	p-value ($\alpha = 0.05$)
CCD7	MK11/3-19	T0 vs. T1	5.37	2.43	1.25	0.06
		T1 vs. T2	0.19	-2.37	1.25	0.06
	MK11/3-66	T0 vs. T1	5.11	2.35	1.07	0.03*
		T1 vs. T2	0.41	-1.27	1.06	0.23
CCD8	MK11/3-19	T0 vs. T1	3.74	1.90	1.73	0.27
		T1 vs. T2	0.85	-0.24	1.73	0.89
	MK11/3-66	T0 vs. T1	5.09	2.35	2.09	0.27
		T1 vs. T2	1.12	0.16	2.09	0.94
MAX2	MK11/3-19	T0 vs. T1	3.36	1.75	1.24	0.16
		T1 vs. T2	0.55	-0.86	1.24	0.49
	MK11/3-66	T0 vs. T1	2.07	1.05	1.10	0.34
		T1 vs. T2	0.63	-0.66	1.09	0.55
BRC1	MK11/3-19	T0 vs. T1	3.55	1.83	1.38	0.19
		T1 vs. T2	0.39	-1.35	1.38	0.33
	MK11/3-66	T0 vs. T1	2.34	1.22	0.94	0.20
		T1 vs. T2	0.53	-0.90	0.93	0.33

Discussion

The shoot branching characteristic of selected genotypes of the MK11/3 population is partly preserved during *in vitro* culture

In spite of the fact that the genotypes MK11/3-19 and -21 were weakly branched (18.3 % and 20.6 %, respectively) and the genotypes MK11/3-66 and -104 were strongly branched (82.3 % and 78.9 %, respectively) after flowering, branching phenotypes changed drastically during *in vitro* cultivation. Any differences between the intact plantlets of the analysed genotypes were hardly detectable indicating an effect of the environment on the trait. The mean

percentage of outgrown side shoots was 3.3 % for MK11/3-19, 3.1 % for MK11/3-21, 11.2 % for MK11/3-66 and 7.7 % for MK11/3-104. This might be due to the limited growth in the culture vessels and the physiological difference between the vegetatively grown plantlets of the *in vitro* culture that lack the generative phase of the greenhouse plants. Nevertheless, MK11/3-66 was still significantly more branched than MK11/3-19 and -21 and even for MK11/3-104 a higher branching rate was still detected than for MK11/3-19 and -21.

All of the analysed genotypes reacted on decapitation with an enhanced outgrowth of side shoots during the *in vitro* culture. This is to be expected because the removal of the shoot apical meristem brakes the apical dominance (Thimann and Skoog, 1933) and side shoots grow out to fulfil the life cycle of a plant. After decapitation there was a significant difference between the genotypes MK11/3-21 and MK11/3-104. But MK11/3-66 was not distinguishable from the weak branched genotypes MK11/3-19 and MK11/3-21 any longer. However, the shoot branching phenotype of all tested genotypes was highly variable among the replicates.

The synthetic SL analogue CISA-1 effectively inhibits shoot branching in chrysanthemum

Recently, SL was characterised as a new phytohormone, which controls the outgrowth of side shoots and thus the breaking of bud dormancy (Gomez-Roldan *et al.*, 2008; Umehara *et al.*, 2008). Natural SLs are composed of a tricyclic lactone (A-, B- and C-rings) that are connected by an enol ether bridge to a D-ring. The structural features that are generally regarded as bioactive, are the lactone D-ring connected to an unsaturated ester or ketone (Mangnus and Zwanenburg, 1992; Zwanenburg *et al.*, 2009; Boyer *et al.*, 2012). According to these findings Rasmussen *et al*. (2013) synthesized the synthetic, fluorescent SL analogue CISA-1 and it was effective in inhibiting shoot branching in *A. thaliana.*

Therefore, we tested the activity of CISA-1 on shoot branching of intact and decapitated chrysanthemum plantlets *in vitro*. In both treatments, CISA-1 significantly decreased shoot branching in the chrysanthemum. However, the tested genotypes of the MK11/3 population did not react on CISA-1 to the same extent. This is supported by our results with intact and decapitated plantlets of the

genotypes MK11/3-19 and -66 that were treated with different concentrations of CISA-1. While intact plantlets of MK11/3-19 did not react on the CISA-1 treatment, intact plantlets of MK11/3-66 reacted with a decrease in branching with all of the tested dosages of CISA-1. Therefore, it could be possible that these genotypes differ in their SL household explaining their different shoot branching phenotypes. MK11/3-19 might process more bioactive SL than MK11/3-66. Indeed, we are not able to directly determine the SL content in these plants.

The pattern was different after decapitation. Both of the genotypes revealed the same increased branching phenotype in the control group (0 µM CISA-1). Neither the plantlets of MK11/3-19 nor of MK11/3-66 did react on the supply with 0.5 µM and 2.5 µM CISA-1. Only the highest dosage of CISA-1 decreased the number of outgrown side shoots in both of the tested genotypes. However, the genotypes did not differ in their shoot branching phenotype at this concentration. The reaction of the genotypes towards the treatment indicates that the main effect on the outgrowth of side shoots is due to the loss of the primary auxin source after decapitation because the outgrowth of side shoots is not completely inhibited even under the highest dosage of CISA-1. The results of Liang *et al*. (2010) also indicate that a competing auxin source was needed in addition to SL to inhibit bud outgrowth in the chrysanthemum. However, we are aware that these results were derived from a limited number of plants and limited sources of CISA-1. Thus, our results highlight the importance of SL on the regulation of bud outgrowth, but also indicate that there might be other factors involved.

Sequence analysis indicates the identification of the chrysanthemum *CCD*7 gene

Based on the sequence information of already characterised *CCD*7 genes of other plant species we were able to identify the CDS of a putative chrysanthemum orthologue after RACE PCR. The predicted amino acid sequence displayed a high similarity with the putative CCD7 of the close relative *A. annua* and was grouped among other functional CCD7 in a phylogenetic analysis. Therefore, there is a high chance that we identified the *CCD*7 gene of chrysanthemum.

However, we are aware that the analysis on the sequence level alone is not sufficient to prove the function of a gene. To test its function the gene could be

knocked-out or a construct with the gene of interest could be used to complement a mutated phenotype. Currently, the complementation of *MAX*3/*CCD*7 mutant lines of *A. thaliana* with the CDS of the putative CCD7 orthologue of chrysanthemum is in progress.

Expression analysis of SL pathway genes and *BRC*1

We analysed the expression of the SL pathway genes *CCD*7, *CCD*8 (Liang *et al.*, 2010) and *MAX*2 (Dong *et al.*, 2013) and the *BRC*1 (Chen *et al.*, 2013) gene of chrysanthemum in two different genotypes (MK11/3-19 and MK11/3-66) before and after decapitation. We also compared the expression of these genes between roots and stem segments with nodes because *CCD*7 and *CCD*8 should be mainly active in the roots based on grafting experiment with *A. thaliana* (Booker *et al.*, 2005) and *P.* × *hybrida* (Foo *et al.*, 2005). While *CCD*7 was stronger expressed in the roots of both genotypes, *CCD*8 transcripts were more abundant in the stem in accordance to the results of Liang *et al*. (2010). The *MAX*2 gene expression was higher in stem fragments with nodes and the expression of the *BRC*1 gene was dramatically higher in the stem, which is in agreement with former results (Dong *et al.*, 2013; Chen *et al.*, 2013).

As the shoot branching phenotype of intact *in vitro* plantlets differed between the genotypes MK11/3-19 and -66, we compared the expression of the candidate genes between them before decapitation. The transcripts of all genes were more abundant in the genotype MK11/3-66 (except of *MAX*2 in stems), although this genotype showed more branching. Combining this finding with the results of intact plantlets during *in vitro* culture, it could be possible that the SL pathway of the genotype MK11/3-66 is less effective than the SL pathway of MK11/3-19.

The decapitation of *in vitro* plantlets resulted in a dramatic change in the expression of all of the analysed candidate genes. In both of the genotypes, the expression of each gene is strongly reduced 12 h after decapitation. The transcript reduction of the SL pathway genes should decrease the amount of bioactive SL in the nodes and therefore reducing the potential of SL to inhibit bud outgrowth. Furthermore, the transcription factor BRC1 directly inhibits branching and bud outgrowth is promoted, if the expression of *BRC*1 is reduced (Aguilar-Martinez *et al.*, 2007; Braun *et al.*, 2012, Dun *et al.*, 2013). One week after decapitation all

tested genes showed an increase in expression indicating the acquisition of new shoot branching inhibitory signals. Indeed, during this time axillary buds are activated and start to grow out. The meristems of these side shoots compensate the loss of the shoot apical meristem and establish a new kind of apical dominance because they compete with each other to release their auxin into the stem (Crawford *et al.*, 2010; Shinohara *et al.*, 2013).

Conclusion

The *in vitro* cultivation of selected genotypes of the MK11/3 population revealed similar shoot branching phenotypes as under greenhouse conditions. Therefore, shoot branching is dependent on the genetics of a chrysanthemum plant. Shoot branching itself is regulated by the phytohormone SL and our study shows that the synthetic SL analogue CISA-1 is effective in inhibiting the outgrowth of axillary buds. Several genes of the chrysanthemum SL pathway have already been characterised. Here, we present sequence information for the putative, yet unknown *CCD*7 gene of chrysanthemum to enhance our knowledge about this pathway. However, the functional conformation of this newly isolated gene is still under progress.

Our results not only highlight the importance of SL on shoot branching in the chrysanthemum, but also indicate that the whole process is influenced by additional factors.

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6 General discussion

Four different studies (Chapter 2, 3, 4 and 5) with various individual focuses were conducted within this thesis on the genetics and genomics of chrysanthemum. The main findings of these studies have already been discussed directly in the corresponding chapter. However, more generalised conclusions and implications are drawn connecting different issues of this thesis in the following. Furthermore, an outlook for the rest and unresolved questions based on the results of this study is given.

6.1 Prevailing polysomic inheritance detected in chrysanthemum

In contrast to cytological studies (Dowrick, 1953; Watanabe, 1977; Li *et al.*, 2011) the analysis of molecular markers in the chrysanthemum MK11/3 population revealed a mainly polysomic inheritance with a random assortment of homologues (Chapter 2). However, there are a few loci showing disomic inheritance as well, which may be due to a partial preferential pairing of chromosomes. Thus, our study suggests to classify chrysanthemums as segmental allopolyploids.

Not only the analysis of molecular marker data, but also the investigation of several important phenotypic traits, such as plant height or flower size, in the MK11/3 population supports this hypothesis because all of the traits were continuous and close to the normal distribution (Chapter 4). If chrysanthemums are strictly allopolyploid, these traits should rather display discrete phenotypic classes because in allopolyploids the alleles do not freely recombine with each other and some of them are never inherited together. This leads to the occurrence of less phenotypic classes. Thus, the phenotypic distributions with continuous variation are consistent with the detected free combination of most marker alleles (Chapter 2).

In this study, several QTLs for important ornamental traits were detected (Chapter 4). For instance, the combined effect in an ANOVA of seven QTLs for the flower size explained 65 % of the total phenotypic variance in the MK11/3 population. However, no single marker explained all of the variation in a given

trait. Based on its polysomic inheritance the characteristic of a monogenic trait in the chrysanthemum is at least affected by the interaction of six homologues loci. The QTL analysis clearly shows that the investigated traits are controlled by more than one locus and are likely polygenic (Chapter 4). The occurrence of polygenes is in agreement with the complex inheritance in a segmental allohexaploid.

6.2 The role of strigolactone in the control of shoot branching in chrysanthemum

In the following the role of the phytohormone strigolactone (SL) on shoot branching in chrysanthemum is considered. Mainly, the Chapters 4 and 5 are discussed and their results are placed in context with each other. To measure the branching propensity of the plants, the ratio of formed side shoots to the number of nodes was estimated. Therefore, the percentage of outgrown side shoots represents the shoot branching trait here.

6.2.1 Characterisation of the putative *CCD*7/*MAX*3 gene of chrysanthemum

The CCD7 protein asymmetrically cleaves the 9, 10 double bond of multiple carotenoid substrates (Schwartz *et al.*, 2004) and is part of the pathway leading to the phytohormone SL. In this study, the putative orthologue of the *CCD*7/*MAX*3 gene was identified in the chrysanthemum (Chapter 5). Analysis of the nucleotide and the predicted amino acid sequence indicate a strong homology with the *MAX*3 gene of *A. annua* and homology with other characterised *CCD*7/*MAX*3 genes of *A. thaliana* and tomato (Chapter 5). However, the sequence information on its own does not fully characterise and prove the function of this candidate gene.

As the chrysanthemum has a hexaploid genome, six homologous loci of this gene are expected. Currently, only one possible orthologue has been characterised by the sequence analysis, but the data indicates the presence of additional ones. For instance, three orthologues of the *CCD*8/*MAX*4 gene (Liang *et al.*, 2010) and the *MAX*2 gene (Dong *et al.*, 2013) were identified in the chrysanthemum. Thus, the copy number of the putative *CCD*7 gene of chrysanthemum should be clarified by southern blot analysis.

Moreover, the functionality of the putative *CCD*7 orthologue has to be proven by knocking-out the gene of chrysanthemum or complementing *CCD*7 mutants of other species. There are publicly available *CCD*7 mutants of *A. thaliana*, which can be complemented by *Agrobacterium tumefaciens* mediated transformation. Therefore, the CDS of the putative *CCD*7 orthologue of chrysanthemum was transferred to a cloning vector for *A. tumefaciens* and these bacteria were subsequently used to transform the mutants of *A. thaliana*. Currently, seedlings of these plants are screened for positive transformation events by applying the herbicide Basta® because the cloning vector carries the *bar* gene as a selection marker. No positively transformed plants are yet identified and this study is still in progress to completely confirm the identification of the *CCD*7 gene of chrysanthemums.

6.2.2 Analysis of candidate genes with a known function in the regulation of shoot branching

The SL pathway genes *CCD*7, *CCD*8 and *MAX*2 and the *BRC*1 gene of chrysanthemum were screened for polymorphisms at the DNA level in a collection of chrysanthemum varieties and the biparental F1 population MK11/3 as well. In both populations marker alleles for all the analysed candidate genes (except *MAX*2 in the MK11/3 population) were identified that were significantly associated with shoot branching (Chapter 4). In contrast to the analysis of AFLP® markers (Chapter 4) the effect of the marker alleles of these genes on shoot branching could be confirmed within the two independent populations. This cross-validation highlights the fundamental role of the SL pathway and related genes (*BRC*1) on the inheritance and characteristic of shoot branching in the chrysanthemum.

However, none of the analysed genes explained the complete phenotypic variation within the populations under investigation. For instance, in the MK11/3 population the combined effect of all significantly associated markers of these genes was 41 % of the total phenotypic variance (Chapter 4). If the marker effects are not only added together but analysed using an ANOVA with interactions, then they determine 39 %. Nevertheless, this assumption is simplified and might overestimate the effects of the markers on the trait because the individual marker alleles are considered to be independent from each other and completely additive.

In reality the included markers might detect different alleles of the same gene or the causal relation of these QTLs might not exist. Thus, the effects of the markers would not just be additive.

Although the detected markers account to a great extent for the variation in shoot branching, this approach might not have detected all possible marker alleles associated with these candidate genes. Additionally, the SL pathway genes *P*450/*MAX*1 (acting downstream of *CCD*7 and *CCD*8; Ferguson and Beveridge, 2009; Kohlen *et al.*, 2011) and *DWARF*27 (acting upstream of *MAX*1; Sato *et al.*, 2005; Bennett *et al.*, 2006) and the SL signalling gene *DWARF*14/*DAD*2 (Arite *et al.*, 2009; Hamiaux *et al.*, 2012) are still not characterised in the chrysanthemum. Therefore, these and other genes related to this pathway might contribute to the phenotypic variation in shoot branching as well. Furthermore, the environment likely affects the shoot branching trait because shoot branching of the selected genotypes of the MK11/3 population was different between greenhouse and *in vitro* culture. It would have been possible to evaluate the environmental effect on the trait by planting the MK11/3 population at different locations. This was not done because cut chrysanthemums are mostly cultivated under controlled greenhouse conditions, which should not vary that much between locations, and the cultivation at different locations requires equipment and staff causing additional costs.

The expression of the aforementioned genes (*CCD*7, *CCD*8, *MAX*2 and *BRC*1) was also analysed in this thesis (Chapter 5). After decapitation the expression of these genes was dramatically reduced, which is in agreement with the profound outgrowth of side shoots (Chapter 5). Thus, the expression of these genes could allow changes to shoot branching in the chrysanthemum after decapitation, although a study in pea describes apical dominance and the SL pathway as distinct mechanisms for the regulation of bud outgrowth (Ferguson and Beveridge, 2009).

The comparison of the expression of these candidate genes between a weak and a strong branched genotype did not reveal any significant differences (Chapter 5). Moreover, the transcripts of the candidate genes were more abundant in the stronger branched genotype. Hence, their phenotypic difference is not due to the expression differences of the analysed SL pathway genes and *BRC*1. Other possible

explanations are post transcriptional regulation, more effective proteins due to DNA/protein sequence differences of the weak branched genotype or other yet undetected factors.

6.2.3 Impact of SL on shoot branching in chrysanthemum

As discussed before the candidate gene analysis of SL pathway genes revealed significant marker associations with shoot branching in chrysanthemum. In order to investigate the physiological reactions on SL *in vitro* plantlets of the MK11/3 population were treated with the synthetic SL analogue CISA-1 (Rasmussen *et al.*, 2013).

In general, not only intact but also decapitated plantlets showed less outgrown side shoots after the treatment with 5 µM CISA-1 (Chapter 5). Thus, it seems to be biologically active in chrysanthemum. Lower concentrations of CISA-1 (0.5 µM and 2.5 µM) also reduced shoot branching in intact plantlets of the strong branched genotype MK11/3-66, while CISA-1 had no effect on intact plantlets of the weak branched phenotype MK11/3-19 (Chapter 5). Therefore, it could be that these genotypes differ in their bioactive SL level or their sensitivity to the reception of SL; after the application of CISA-1 this difference became neglectable. Indeed, the SL content has to be directly determined in the plant tissue under investigation to clarify this, but the detection of SLs in the plant material is not trivial and requires a lot of technical equipment and chemical knowledge (Sato *et al.*, 2005; Gomez-Roldan *et al.*, 2008; López-Ráez *et al.*, 2008; Vogel *et al.*, 2010; Kohlen *et al.*, 2011).

However, after decapitation the reaction to CISA-1 was not persistent. First of all, the genotypes MK11/3-19 and -66 showed no difference in shoot branching after decapitation (Chapter 5). Thus, decapitation seems to be a severe change in the physiology of the chrysanthemum plants. Both of the genotypes showed less shoot branching with the highest concentration of 5 µM of CISA-1, whereas the lower ones (0.5 µM and 2.5 µM) were not effective (Chapter 5). The extent of the reduction in branching with CISA-1 was not different between the two MK11/3 genotypes. Furthermore, the plantlets of both genotypes still showed the outgrowth of side shoots. Liang *et al*. (2010) also reported that the efficient inhibition of bud outgrowth by SL is dependent on the presence of a competing auxin source. Hence, at least one side shoot has to grow out after decapitation and

to establish a new auxin source with its developing axillary meristem, so that the outgrowth of other buds is inhibited.

From a breeders point of view it seems to be possible to alter the shoot branching habit of chrysanthemums by the SL pathway. However, the experimental setup of this thesis did not detect whether the branching genotypes really differ in their intrinsic SL content causing the phenotype. Nevertheless, favourable alleles of the candidate genes could be combined to improve the branching trait because the effect of a single allele is rather low. The analysis of these alleles results in excessive costs because laboratory equipment and experienced laboratory assistants are required. Hence, these markers could be most effectively used to select parents for segregating populations in which favourable alleles are maximised with respect to parental combinations.

In summary, the results of this study demonstrate that SLs are promising candidates to alter the shoot branching habit of chrysanthemums. However, SLs interacts with other phytohormones, such as auxin (Bennett *et al.*, 2006; Crawford *et al.*, 2010; Shinohara *et al.*, 2013) and cytokinin (Ferguson and Beveridge, 2009; Braun *et al.*, 2012; Dun *et al.*, 2013), in the regulation of shoot branching. Consequently, the analysis of SL on its own does not totally explain the variation observed in shoot branching in chrysanthemum and there seem to be several other factors involved.

6.2.3 Additional QTLs for shoot branching

In this thesis two different approaches were applied for the identification of markers associated with shoot branching that might not be related to the SL pathway.

Two types of chrysanthemum populations, a collection of 81 varieties and a biparental F1 population of 160 individuals, were genotyped with AFLP® markers. In a genome wide association study (GWAS) one of the 1000 AFLP® markers was associated with the percentage of outgrown side shoots being the key measure for shoot branching (Chapter 4).

Additionally, three AFLP® markers were identified being associated with the percentage of outgrown side shoots in the MK11/3 population (Chapter 4). The

effects of these QTLs ranged from 7 to 10 % and the combined effect (ANOVA) of them was 17 % of the total phenotypic variance.

The detected loci partly contribute to the characteristic of shoot branching in the MK11/3 population. With the results of the candidate gene approach the combined effect of all detected QTLs was 56 %, which explain a lot of the phenotypic variation in shoot branching. Nevertheless, there should be additional loci present in the genome of chrysanthemum and these results indicate a polygenetic mechanism for the control of shoot branching in the chrysanthemum. This complex pattern is in contrast to the regulation of shoot branching in the sunflower (*Helianthus annuus*), which is a close relative of chrysanthemum. Rojas-Barros *et al.* (2008) identified only one single dominant apical branching gene in the cultivated sunflower. The functional characterisation of this gene could be helpful to screen the chrysanthemum genome for orthologues that might affect branching as well, although the results of this thesis call the existence of such a locus for chrysanthemum into question.

6.3 Outlook

With this thesis a considerable progress was achieved towards an improved understanding of genetic and genomic processes in the complex polyploid ornamental chrysanthemum. Especially, the role of the phytohormone SL in the regulation of shoot branching was investigated. The validation and interpretation of the observed results reveals the rest and new questions that could be addressed in the future. Some of these are introduced in the following section.

6.3.1 Which loci are detected by the AFLP® markers and can these ones be transferred to other populations?

Based on the results of Chapter 4 and 5 it is very likely that shoot branching is affected by various factors in the chrysanthemum. Some of them might have already been located by the associated, anonymous AFLP® markers. However, the applicability of these markers is limited because they are multilocus and anonymous markers. They can be transferred into sequence specific markers, but this is laborious because an AFLP® fragment might consist of several different genomic regions of the same size. Furthermore, the detected AFLP® polymorphism

has to be preserved on the sequence specific level in other populations of chrysanthemum to further validate the marker-trait association.

Additionally, not only the transformation of the AFLP® markers is needed, but also more closely linked markers for the detected loci should be identified. With the help of these markers it could be possible to isolate the genomic region which causes the detected effect on shoot branching. Thus, the sequence information of this region could be used to characterise new candidate genes that contribute to the shoot branching trait in chrysanthemum.

6.3.2 Are further candidate genes for shoot branching accessible in the chrysanthemum genome?

As sequencing costs fall rapidly and new technologies and strategies for high-throughput sequencing are permanently developed, the generation of valuable sequence data is at our fingertips. For instance, a comparative transcriptome analysis between the two different branching genotypes MK11/3-19 and -66 might reveal differentially expressed genes which cause the phenotypic difference of these genotypes. Those new sequence information for chrysanthemum can be screened for further candidate genes regulating shoot branching, such as the SL pathway genes *DWARF*27 (Sato *et al.*, 2005; Bennett *et al.*, 2006) and *P*450/*MAX*1 (Ferguson and Beveridge, 2009), the SL transporter *PDR*1(Kretzschmar *et al.*, 2012), *SPS* (Tantikanjana *et al.*, 2001) or *REV* (Otsuga *et al.*, 2001).

Moreover, the sequencing of the whole genome of sunflower is in progress (https://sunflowergenome.org/). As sunflower is also a member of the *Asteraceae* family, this sequence information can be used to screen for homologues of known candidate genes regulating shoot branching. This information might be helpful to characterise those genes in chrysanthemum as well due to synteny between the sunflower and the chrysanthemum genome.

6.3.3 Is it possible to measure the SL content in a chrysanthemum plant?

To further validate if the different branching phenotypes of the MK11/3 population are directly caused by varying phytohormone contents, it would be necessary to determine the amount of SLs and probably other phytohormones, such as auxin or cytokinin, in these plants.

Several research groups (e. g., Sato *et al.*, 2005; Gomez-Roldan *et al.*, 2008; López-Ráez *et al.*, 2008; Vogel *et al.*, 2010 or Kohlen *et al.*, 2011) published detection methods for SL. Plant extracts are dissolved with organic solvents (e. g., acetone or methanol) and purified via high-performance liquid chromatography (HPLC). Subsequently, the components of the extracts are identified using mass spectrometry. Hence, a lot of technical equipment and chemical knowledge is required for the direct quantification of SL. In contrast to SL, auxin and cytokinin contents were measured via enzyme-linked immunosorbent assay (ELISA) in chrysanthemums after the purification of plant extracts (Jiang *et al.*, 2010; Jiang *et al.*, 2012). Such an analysis is more convenient and capable of high throughput analysis and would be beneficial for the detection of SLs as well.

In addition to the direct measurement, bioassays are reported for the indirect determination of SL contents. As SLs were initially identified as germination stimulants for parasitic plant seeds of the genus *Striga* or *Orobanche* (Cook *et al.*, 1966; Cook *et al.*, 1972; Siame *et al.*, 1993), a common method is the use of a germination assay to detect SLs (Chae *et al.*, 2004; Matusova *et al.*, 2005). However, seeds of the genus *Striga* or *Orobanche* are not easily obtained and they require preconditioning for a certain period of time at a suitable temperature before becoming responsive to germination stimulants. Moreover, a SL source is needed as a positive control for germination. In the first own test, no results were achieved with seeds of *Orobanche minor.*

SL was also unveiled as a branching factor in symbiotic arbuscular mycorrhizal (AM) fungi. Thus, Akiyama *et al.* (2005) describe the use of spores of AM fungi in a bioassay. If SLs are present, hyphae show increased branching. However, to obtain and preserve a culture of AM fungi is complex and laborious due to the need of the co-cultivation in symbiosis with a host plant.

It was reported that SLs negatively regulate adventitious root formation (Rasmussen *et al.*, 2012), so an adventitious rooting assay was developed for *A. thaliana* (Sorin *et al.*, 2006) and pea (Rasmussen *et al.*, 2012). Plant seeds are sown *in vitro* and germinate in the dark. Seedlings are further grown in the dark and are subsequently transferred to light, in long-day conditions. After one week the

number of adventitious roots can be recorded. However, a SL source is needed again as a positive control.

To summarise, although several methods for measuring SLs have been reported, all of them suffer from their complexity and the need of available SL resources. Nonetheless, the development of an ELISA for SLs could be an easy and reliable detection method.

6.3.4 Implications for the breeding process of chrysanthemums

Although the results of this study highlight the role of SLs in the regulation of bud outgrowth in chrysanthemum, the causing effect of SL is not completely proven unless there is no detected direct correlation between the SL content and shoot branching. The single effects of the detected markers of this study are limited because shoot branching and also other important traits seem to be inherited as polygenes. Thus, it would be necessary to combine several favourable alleles to improve these traits. Although these alleles could be freely combined because of the detected hexasomic inheritance, this would require several successive crossings. At this point the application of marker-assisted selection is causing high costs due to the abundant screenings that are also hampered by the complex genetics of chrysanthemums. Additionally, the transfer of the alleles to a homozygous state is unlikely, as chrysanthemums are strictly outcrossing and show inbreeding depression.

Consequently, the precision breeding for polygenic traits, such as shoot branching or flower size, in a polyploid genome is less effective and costly. The meaningfulness of the shoot branching phenotype of a single plant should be high because the effect of single alleles on the trait is limited. Furthermore, the aesthetic value of an ornamental is determined by numerous traits (e. g., flower size, colour or shape, growth habit, leaf shape etc.). Focusing on a single trait is not effective to develop a new marketable variety. Hence, precision breeding in chrysanthemums is limited to monogenic traits and phenotypic selection should be prioritised in the breeding of chrysanthemums. However, the identified markers of this study could be used to select parents for segregating populations in which favourable alleles are maximised with respect to parental combinations.

In summary, several conclusions for the breeding progress of chrysanthemums might be drawn due to the findings of this study:

i) based on its complex polysomic inheritance the selection of new varieties should be performed with a large number of progenies
ii) the information provided by a single marker is limited in a hexaploid genome and its analysis might be hampered by the complex genome of chrysanthemum making such an analysis costly
iii) the ornamental value of chrysanthemums is composed of several traits and the selection for a single trait or marker alone is not effective for the development of new varieties because the other traits are likely adversely effected
iv) for hexasomic inheritance free recombination occurs between homo- and homoeologous chromosomes; thus, there should be negligible positive or negative correlations between the traits
v) further progress in chrysanthemum breeding could be achieved by crossing genotypes from the extreme ends of the trait distributions
vi) favourable alleles should be enriched in the breeding material
vii) as chrysanthemums are highly heterozygous and display a polysomic inheritance, favourable alleles are difficult to transfer to a homozygous state; chrysanthemums can also show self-incompatibility (Drewlow *et al.*, 1973; Anderson *et al.*, 1992) hampering the development of inbred lines

6.4 Achievements in relation to the set thesis objectives

The seven main objectives of this thesis were previously described in Chapter 1.4. For all stated objectives considerable progress was achieved that provides new insights into the genetics of chrysanthemum and the regulation of shoot branching. Here, the objectives are modified to statements according to their level of achievement that is briefly explained.

i) the analysis of the molecular markers indicates a polysomic inheritance in chrysanthemums, as is characteristic of an autopolyploid

Based on their evolutionary origin and published cytological analyses, cultivated chrysanthemums have been classified as allopolyploid plants. In contrast, Langton (1989) described the hexasomic inheritance of the carotenoid pigmentation in chrysanthemums, as it is typically for autohexaploids. Therefore, more informative methods were used based on molecular marker analyses to resolve this contradiction in this thesis. Most of the applied methods (segregation patterns of SSRs and the ratios for markers linked in coupling to those linked in repulsion) indicated a polysomic inheritance in chrysanthemum, as it is characteristic for an autopolyploid (Chapter 2). However, some markers segregated in a disomic manner and the ratios of marker dosages were not conclusive (Chapter 2). Therefore, the inheritance in chrysanthemum seems to be mainly polysomic with a random assortment of homologues, but there are a few loci with disomic inheritance as well due to a partial preferential pairing of chromosomes. Thus, cultivated chrysanthemums should rather be classified as segmental allopolyploids.

ii) based on multilocus AFLP® marker the overall genetic similarities between the chrysanthemum cultivars are comparatively moderate to high and the relatedness of the cultivars is unstructured

The genetic similarity within the collection of different *C. indicum* hybrid varieties and some related wild species was analysed by applying 448 polymorphic AFLP® markers. Most of the varieties were genetically close to each other, as exemplified by 75 % of all of the pairwise genetic similarities ranging from 0.6 to 0.9, with an average of 0.72 (Chapter 2). However, this moderate or rather high genetic similarity is in contrast to a large phenotypic diversity of the varieties for important horticultural traits. The collection of chrysanthemum varieties remained unstructured because there was a lack of correlation between different horticultural groups as based on their phenotypic traits or the breeding programmes (Chapter 2).

iii) all characterised phenotypic traits are continuous and are only weakly correlated in an association panel and a biparental mapping population

A collection of 81 chrysanthemum accessions was phenotyped for plant height, internode length, number of nodes, number of formed axillary buds, number of

formed side shoots, length of the longest side shoot and inflorescence diameter. All investigated traits varied to a significant extent, but were not always normally distributed (Chapter 4). In contrast, most of the characterised traits (such as flower size, plant height, number of nodes and number of formed side shoots) were normally distributed in the MK11/3 population (Chapter 4). Only weak correlations between traits were observed in the collection of chrysanthemum varieties and in the MK11/3 population (Chapter 4). The most strongly correlated traits were plant height and the length of the longest side shoot. The occurrence of mostly continuous traits is consistent with the free combination of most marker alleles (Chapter 4).

iv) the genome wide association study (GWAS) and the QTL analysis in a biparental mapping population are suitable to detect marker-trait associations in the complex, less characterised genome of chrysanthemum

For this thesis 1000 polymorphic AFLP® markers were analysed in an association panel and 15 marker-trait associations were identified (Chapter 4). This study is the first GWAS to provide a preliminary glimpse into the very complex structure of the chrysanthemum genome and is a useful way of identifying loci for candidate genes for important horticultural characteristics. In addition to the GWAS, 327 polymorphic AFLP® markers were scored in the MK11/3 population. This information was used to detect 17 marker-trait associations for the population by applying a single locus analysis (Chapter 4). The markers significantly associated with important horticultural characteristics in chrysanthemum, which were detected by the GWAS and QTL analysis, explained phenotypic variations ranging from 2 to 34 % (Chapter 4). Because no single marker explained all of the variation in a given trait, it is clear that the analysed traits are controlled by more than one gene and might also be affected by environmental factors (e. g., the difference of the selected genotypes of the MK11/3 population under greenhouse and *in vitro* culture; Chapter 5). To evaluate the environmental effect on the traits it would be necessary to test the MK11/3 population at different locations in the future.

v) marker alleles of candidate genes of the strigolactone pathway and *BRC*1 are significantly associated with shoot branching in both an association panel and a biparental mapping population

Four candidate genes (*CCD*7, *CCD*8, *MAX*2 and *BRC*1) were screened for polymorphisms by SSCP analysis in a collection of different chrysanthemum genotypes and in the MK11/3 population. Marker alleles were identified that were significantly associated with shoot branching and produced variations in the percentage of outgrowth ranging from -12 to 23 % in the association panel and from 6 to 12 % in the biparental mapping population (Chapter 4). The effect of marker alleles from the candidate genes *CCD*7, *CCD*8 and *BRC*1 on shoot branching was confirmed in both of the chrysanthemum populations. Nevertheless, the candidate gene approach might not have detected all possible marker alleles associated with the analysed genes. Furthermore, it is very likely that shoot branching in the chrysanthemum is influenced by additional loci (Chapter 4). In summary, this study highlights the fundamental role of the SL pathway. Moreover, the results indicate that shoot branching in the chrysanthemum has a polygenic inheritance pattern, though other yet unknown factors are also likely involved.

vi) the putative *CCD*7 orthologue of chrysanthemum has been identified based on the nucleotide sequence analysis

The CCD7 protein is part of the pathway leading to the phytohormone SL. Based on the sequence information of already characterised *CCD*7 genes of other plant species the CDS of a putative chrysanthemum orthologue was identified after RACE PCR (Chapter 5). It covered 1851 bp encoding a predicted protein of 616 amino acids (AA). The predicted AA sequence displayed a high similarity with the putative CCD7 of the close relative *A. annua* and was grouped among other functional CCD7 in a phylogenetic analysis (Chapter 5). Transcripts of the putative *CCD*7 gene were more abundant in the roots than in the stem. However, the functionality of the identified gene has still to be confirmed. This is currently done by the complementation of *CCD*7/*MAX*3 mutants.

vii) SL interacts with other factors to control shoot branching in the chrysanthemum

The synthetic SL analogue CISA-1 decreased shoot branching of intact and decapitated chrysanthemum plantlets *in vitro* (Chapter 5). However, the tested different branching genotypes of the MK11/3 population did not react on CISA-1 in the same way. While intact plantlets of the weak branched genotype MK11/3-19 did not react on the CISA-1 treatment, intact plantlets of the strong branched genotype MK11/3-66 reacted with a decrease in branching with all of the tested dosages of CISA-1 (Chapter 5). Therefore, these genotypes might differ in their SL household explaining their different shoot branching phenotypes. However, the transcripts of all genes were more abundant in the genotype MK11/3-66 (except of *MAX*2 in stems, Chapter 5). Thus, the SL pathway of the genotype MK11/3-66 might rather be less effective than the pathway of MK11/3-19.

After decapitation the pattern was different. Both of the genotypes revealed the same increased branching phenotype. The expression of each analysed candidate gene (*CCD*7, *CCD*8, *MAX*2 and *BRC*1) was strongly reduced 12 h after decapitation (Chapter 5). The transcript reduction of the SL pathway genes should decrease the amount of bioactive SL in the nodes and therefore reducing the potential of SL to inhibit bud outgrowth. Only the highest dosage of CISA-1 decreased the number of outgrown side shoots in both of the tested genotypes after decapitation, but the genotypes did not differ in their shoot branching phenotype at this concentration (Chapter 5). The reaction of the genotypes towards the treatment indicates that the main effect on the outgrowth of side shoots is due to the loss of the primary auxin source after decapitation (apical dominance), as the outgrowth of side shoots is not completely inhibited even under the highest dosage of CISA-1. Thus, the results demonstrate an important role of SL on the regulation of bud outgrowth, but other factors seem to be involved as well.

In conclusion, the major thesis objectives were achieved. Novel information about shoot branching was obtained and the data indicate a quantitative characteristic of this trait. Its detected variation is influenced by candidate genes of the SL pathway, although there are additional loci contributing to shoot branching that are not related to this pathway. Additionally, by the detection of polysomic inheritance in chrysanthemum hybrids they should be considered as segmental allohexaploids, which requires different concepts for genetic analyses. All the

information may serve as a basis for future breeding programmes and future studies in *Chrysanthemum indicum* hybrids.

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Appendices

Supporting information

Table S3.1: Summary of the chrysanthemum genepool. Abbreviations: A) Flower: A (anemone), B (ball formed),D (decorative), E (single), G (filled), HG (half filled), P (pompon) and S (spider type); B) Flower size: s (small), m (middle), b (big); C) Vigour: 1 (slowly growing), 2 (middle growing), 3 (strong growing)

Variety	ID	Origin	Breeder	Class	Flower	Flower size	Flower Colour	Vigour	Branching rate
Ping Pong golden	2510		Dekker	Garden	P	s	yellow	3	49.3
Boris Becker	5093		Dekker	Cut	B	s	white	3	30.5
Moroni	5309		Brandkamp	Pot	A	s	purple	1	83.7
Pera	5312		Brandkamp	Cut	E	s	pink	1	79.3
Ipsili	5468		Brandkamp	Cut	E	s	yellow/red	1	79.9
Elassa	5570		Brandkamp	Cut	E	m	purple/white	2	87.7
Heidi gelb	8215		Yoder	Cut	G	s	yellow	2	
Boula	10104	DE	Deliflor	Cut	G	m	white	3	44.2
Anastasia	10106	DE	Deliflor	Cut	S	b	white	2	45.2
Snow Eleonora	10130	DE	Yoder	Cut	A	b	white	3	27.8
Palisade	10135	KE		Cut	B	b	white	2	33
Macaou	12152	LK	Brandkamp	Cut	P	s	yellow	3	44.1
Trentino	13121	LK		Cut	E	s	pink	3	27.6
Dark Flamenco	13370	LK	Yoder	Cut	D	b	purple	3	46.3
Westland Regal	13490	DE	Yoder	Cut	S	b	purple	3	53.7
Stellar Time	15112	KE	Cleangrow	Pot	G	m	white	1	43
Smola	15115	KE	Brandkamp	Pot	G	m	white	1	40.7

Miral	15195	LK	Gediflora	Pot	B	m	white	2	32.2
Melosa	15214	KE	Brandkamp	Pot	E	s	yellow	2	48
Trumpf gelb	15285	LK	Ball / PanAmerican Seed	Pot	D	s	yellow	1	26.9
Dragona	15405	KE	Brandkamp	Pot	E	m	red	1	55.7
Swona	15580	KE	Brandkamp	Cut	G	m	pink/purple	1	64.1
Branices	16153	LK	Brandkamp	Mums	G	s	white	3	
Euro Sunny	16526		Decker	Mums	G	m	yellow	2	62.7
Avignon	18042	DE		Cut	D	b	light pink	3	38.5
Daily Mirror	18060	DE	Rowe/GB	Garden	B	b	purple	3	39
Princess Armgard	18084	KE		Cut	D	b	bronce	3	43.2
Breitner weiß	18096	DE		Garden	D	b	white	3	48.5
King Fisher	20953		Fides	Cut	E	s	purple	3	48
Lexy red	24412		Fides	Cut	P	s	red	3	39.4
Woodpecker Sunny	25840		Fides	Cut	E	m	yellow	2	
Resomee splendid	26046		van Zanten	Cut	A	b	purple	2	64
Anastasia Green	ANSP		Deliflor	Cut	D	b	green	2	53
Donna	AYPD		Yoder	Mums	E	s	yellow	1	
Bronce Eleonora	BREL		Deliflor	Cut	A	b	bronce	2	63
Bradfort rotbronze	BRRO		Deliflor	Cut	D	m	red/bronce	2	79.5
Chry. coccineum	Cc 1	Caucas		Garden	E	s	yellow	1	32
Annecy Yellow	DAY		Deliflor	Cut	S	m	yellow	3	45.8
Deliwind yellow	DDY		Deliflor	Cut	B	s	yellow	2	77.4
Managua	DM		Deliflor	Cut	E	m	orange	1	69
Milano Dark	DMD		Deliflor	Cut	G	m	purple	3	63

Polar	DP		Deliflor	Cut	G	m	white	3	72
Dreamstar Mums Kipli gelb	DRMH		Kientzler	Cut	G	s	yellow	1	
Dreamstar Mums Kipli	DRMK		Kientzler	Cut	G	m	yellow/bronce	1	52
Enjoy-	ENJ		Cleangro	Cut	A	s	bronce	2	74.9
Chr. maximum Ramond	F-236	ESP		Garden	E	NA	white	1	NA
Alto blanc	FAB	F		Cut	G	m	white	1	41.7
Calinda	FC	F		Pot	G	m	yellow	1	40.8
Calypso bronze	FCP	F		Pot	E	m	bronce	1	56.7
Froggy	FF		Fides	Cut	P	s	green	3	37.5
Sir de Louisette Jaune	FLJ	F	GT/Fr.	Cut	G	m	yellow	2	74.4
Ludo orange	FLO	F	Gediflora	Cut	G	b	orange	1	43.2
Lollipop purple	FLP		Fides	Cut	G	s	purple	3	68.3
Fleury Splendid	FLSB	F	Fides	Cut	E	s	purple	2	31.3
Malabar	FM	F	Challet Herault/Fr	Cut	G	NA	purple	1	18.3
Movida Janine	FMJ	F		Cut	G	m	yellow	1	39
Ford	FOR	F	Yoder	Cut	A	s	white/green	3	54.7
Pivatini Purple	FPP		Gediflora	Cut	G	b	purple	2	67.3
Salambo	FS		Pieters Joseph & Luc BVBA	Pot	G	NA	pink	2	23
Toccata Janine	FTJ	F		Pot	G	m	yellow	2	26
Golden Snowdon	GOSN		Frampton Nurseries/GB	Cut	B	b	yellow	2	49.6
Chr. haradijanii Rech. F.	HA2-39	SY		Garden	E	m	yellow	NA	
Chr. x hortorum 'Bienchen'	HB1			Garden	E	s	bronce	1	82.8
Heidi weiß	HEWE		Yoder	Cut	G	s	white	1	

Chr. x hortorum 'Schneewolke'	HS1		Garden	E	s	white	1	
White Spider	JWS	Japan	Cut	S	b	white	2	72.4
Kientzler Rote Wendy	KRW	Kientzler	Cut	G	m	red	1	51.9
Lima rosa/grün	LIRG	Yoder Brothers	Cut	HG	s	pink/green	2	79.9
Edelgard	OED	Brandkamp	Garden	S	m	yellow	2	75
Fellbacher Wein	OFW		Garden	G	m	red	1	74
Gelbe Latriumfant	OGL		Garden	G	m	yellow	2	79.8
Monks Rote	OMR		Garden	G	m	red	2	77.2
Sämling 02	OS02		Garden	E	m	yellow	1	83.8
Sämling 04	OS04	Fides	Garden	G	m	light pink	1	
Samos	SAMO	Deliflor	Cut	A	s	pink	3	50.1
Snowdon weiß	SNWE	Yoder	Cut	G	b	white	3	75.1
Vyking gelb	VYGE	Van Zanten	Cut	E	s	yellow	3	38.6
Mega Time gold	VZMTG	van Zanten	Pot	E	s	yellow	3	88.2
Relinda	VZR	van Zanten	Cut	A	s	white	3	74.3
Yellow Arras	YEAR	Fides or Deliflor	Cut	G	s	yellow	1	31.3
Elmira Dark	YED	Yoder	Cut	G	s	purple	1	59

Supplemental S3.2: Example for the R-code for the bootstrap computation of all of the pairwise genetic distances (Jaccard) for a marker size of 50.

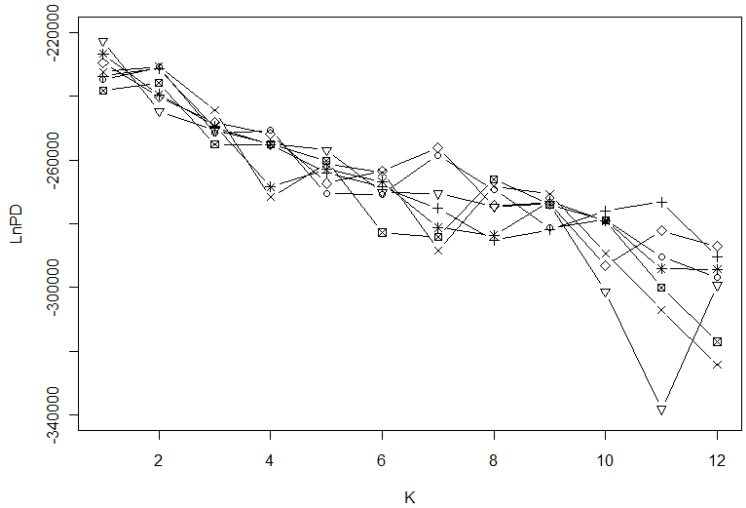
```
#Jaccard Distance method included in the following package

library (proxy)

#Import Data-File

dat <- read.table("")

#Lists to save the output of the bootstrapping

ListMat <- as.list(rep(NA,100))

ListMatDist <- as.list(rep(NA,100))

#Take 50 marker randomly 100 times

for(i in 1:100){

ListMat[[i]] <- dat[sample (dat$Pflanze,50,replace =TRUE),]

ListMatDist[[i]] <- dist(t(ListMat[[i]][-1]), method = "Jaccard")

}

#Stores all pairwise distances of the 100 different data sets

SumDistMat <- matrix(rep(NA, 100*3655), ncol=3655, nrow=100)

for(i in 1:100){

SumDistMat[i,] <- ListMatDist[[i]][1:3655]

}

SumDist <- as.data.frame(SumDistMat)

#Calculation of the CV via mean and standard deviation

m50 <- apply(SumDistMat, MARGIN=2, FUN=mean)

sd50 <- apply (SumDistMat, MARGIN=2, FUN=sd)

cv50 <- sd50/m50*100
```

Supplemental S3.3: Detection of the most likely number of populations K according to three different methods using STRUCTURE 2.3.2.1 with the following settings: admixture model for dominant markers (ploidy level of six)), with a burn-in period of 100000, followed by 300000 iterations. Seven independent runs were accomplished for each K, ranging from 1 to 12. a) method of Pritchard et al. (2000) for each repeated run, b) method of Cockram et al. (2009) for each repeated run exemplified by the different symbols and c) ΔK method of Evanno et al. (2005)

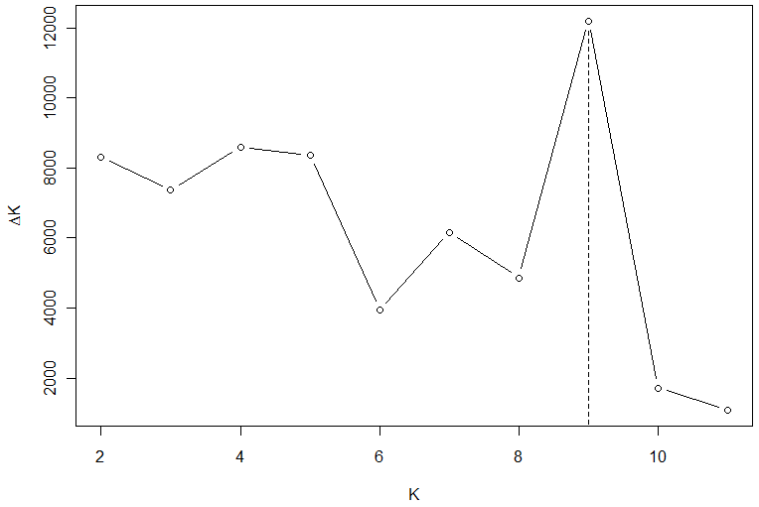
a

K	log P(K|X$_1$)	log P(K|X$_2$)	log P(K|X$_3$)	log P(K|X$_4$)	log P(K|X$_5$)	log P(K|X$_6$)	log P(K|X$_7$)
1	~0	~0	~1	~1	~0	~1	~0
2	~1	~1	~0	~0	~1	~0	~1
3	~0	~0	~0	~0	~0	~0	~0
4	~0	~0	~0	~0	~0	~0	~0
5	~0	~0	~0	~0	~0	~0	~0
6	~0	~0	~0	~0	~0	~0	~0
7	~0	~0	~0	~0	~0	~0	~0
8	~0	~0	~0	~0	~0	~0	~0
9	~0	~0	~0	~0	~0	~0	~0
10	~0	~0	~0	~0	~0	~0	~0
11	~0	~0	~0	~0	~0	~0	~0
12	~0	~0	~0	~0	~0	~0	~0

b



c



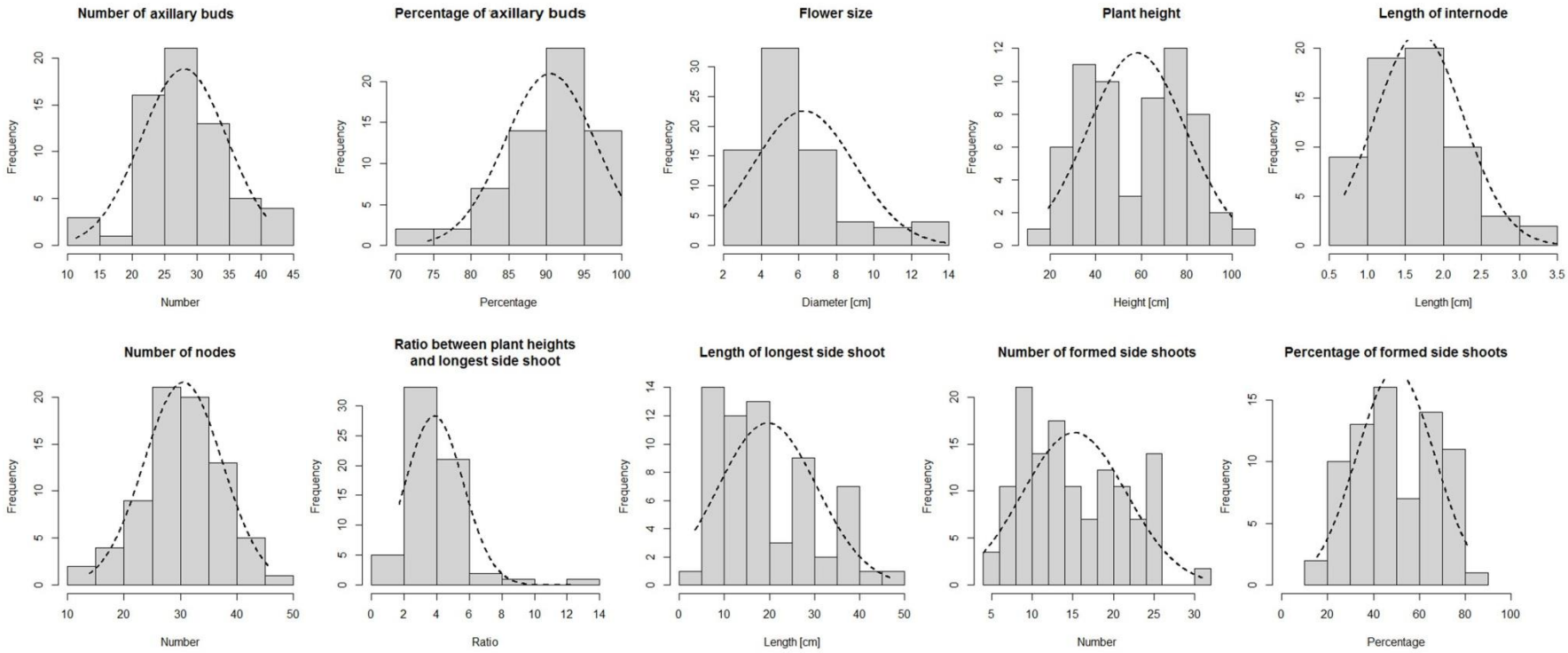


Figure S4.1: Phenotypic distribution of the traits collected for a collection of the chrysanthemum genotypes comprised of 81 accessions. Means for each trait were calculated for three independent clones of each accession. The normal distribution (using the overall mean and standard deviation) for the traits is represented by the dashed line.

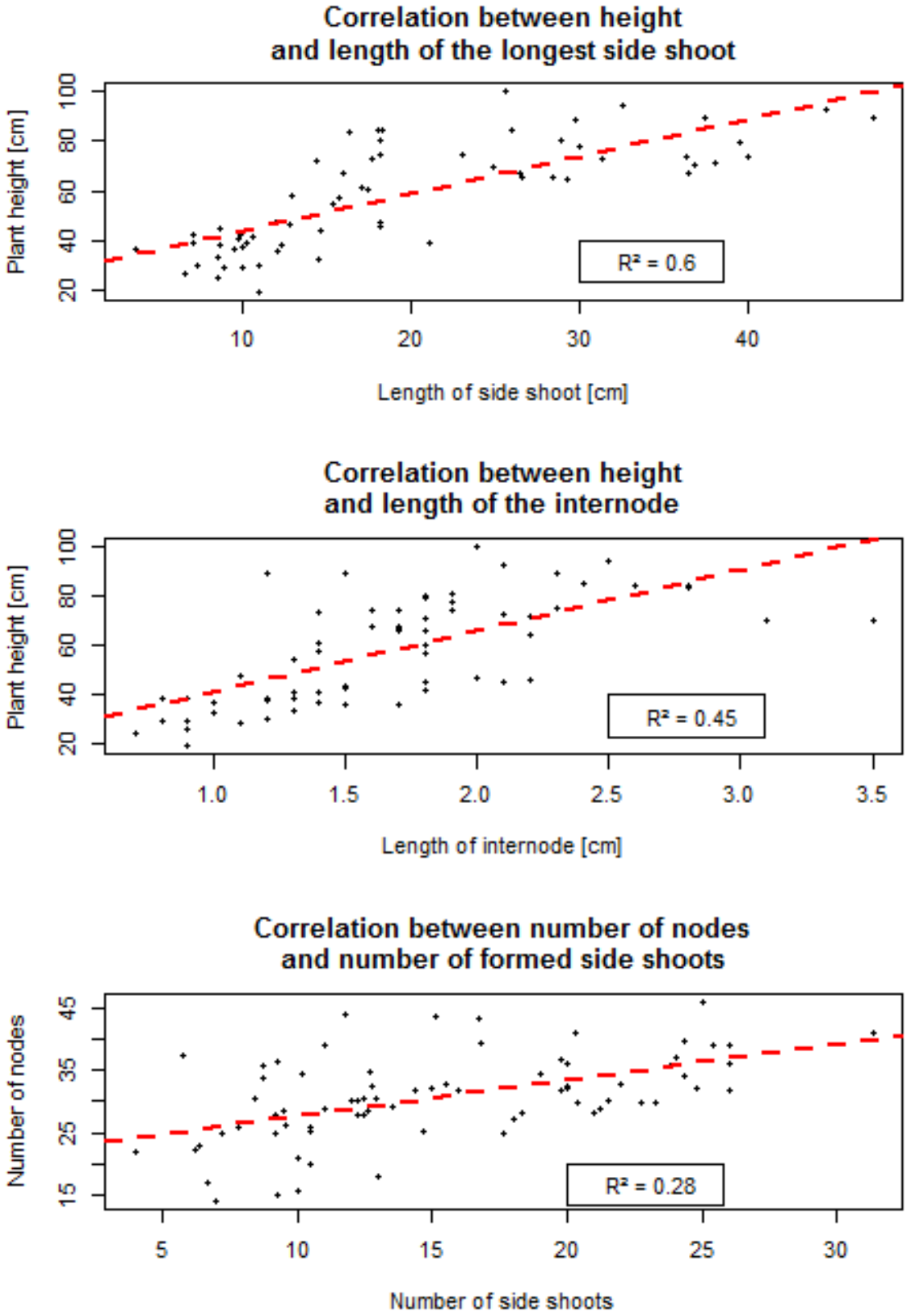


Figure S4.2: Correlations of the traits within the collection of the chrysanthemum genotypes comprised of 81 accessions.

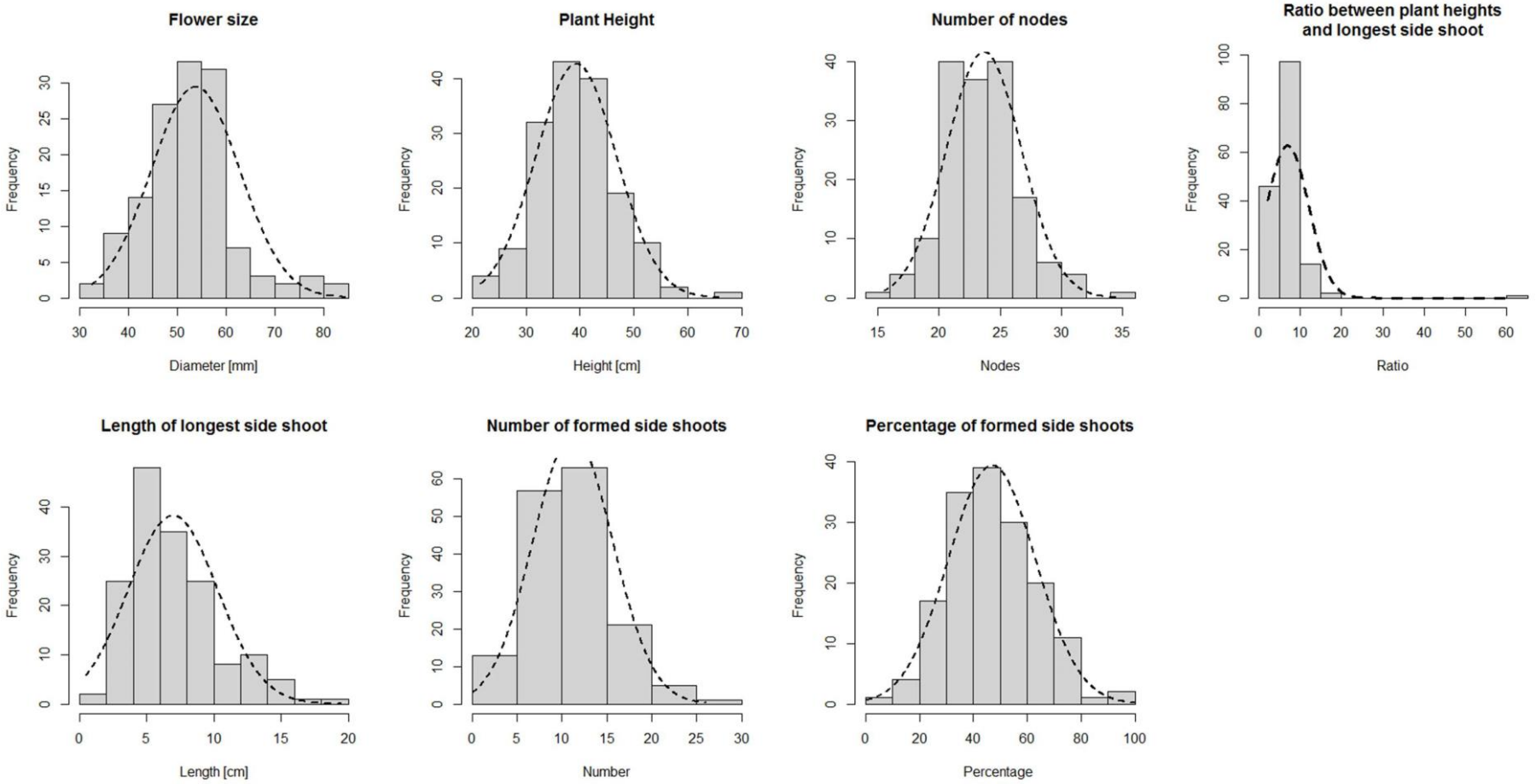


Figure S4.3: Phenotypic distribution of the traits collected for the MK11/3 population comprised of 160 genotypes. Means for each trait were calculated for three independent clones of each genotype. The normal distribution (using the overall mean and standard deviation) for the trait is represented by the dashed line.

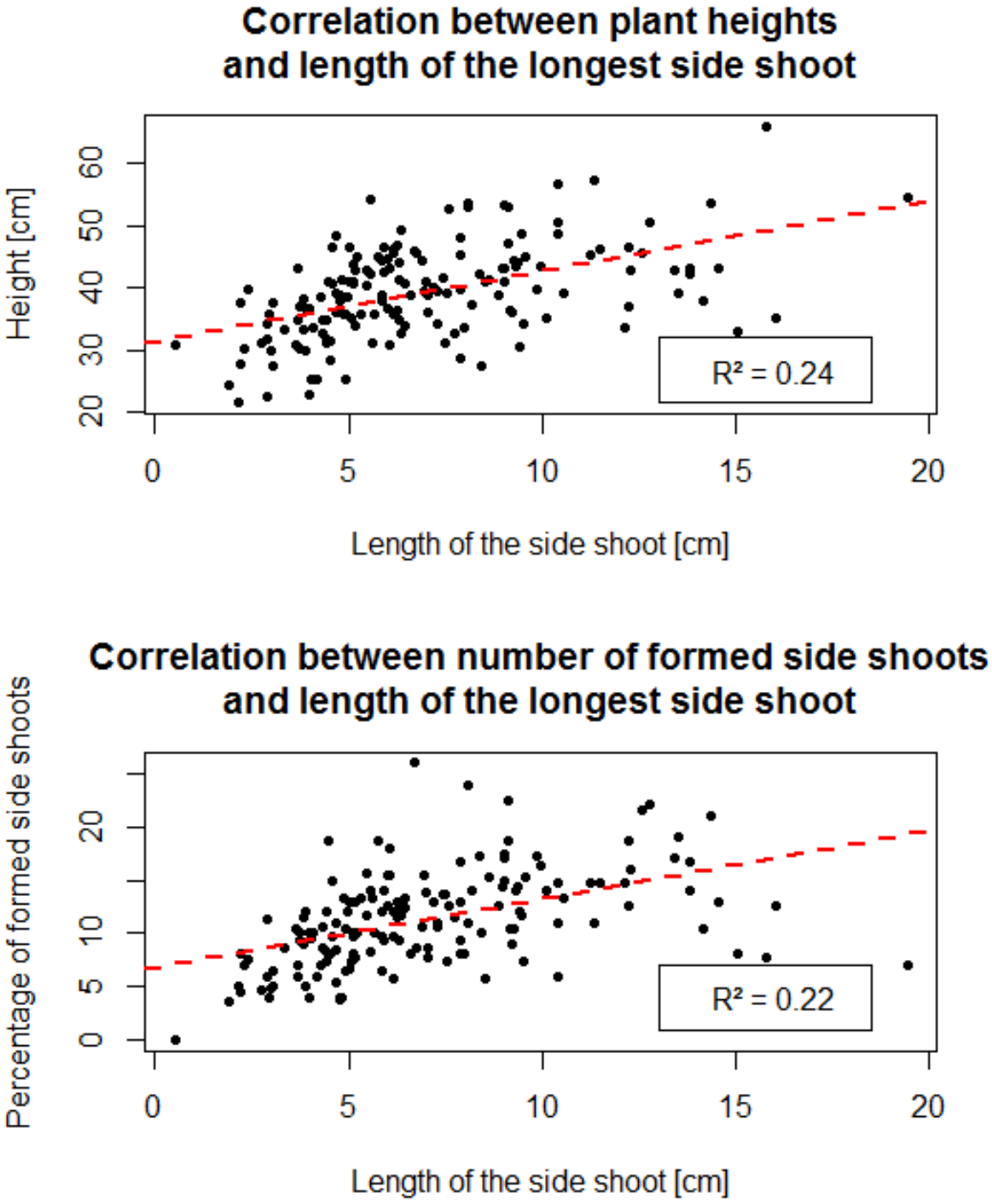


Figure S4.4: Correlations of the traits within the MK11/3 population comprised of 160 genotypes.

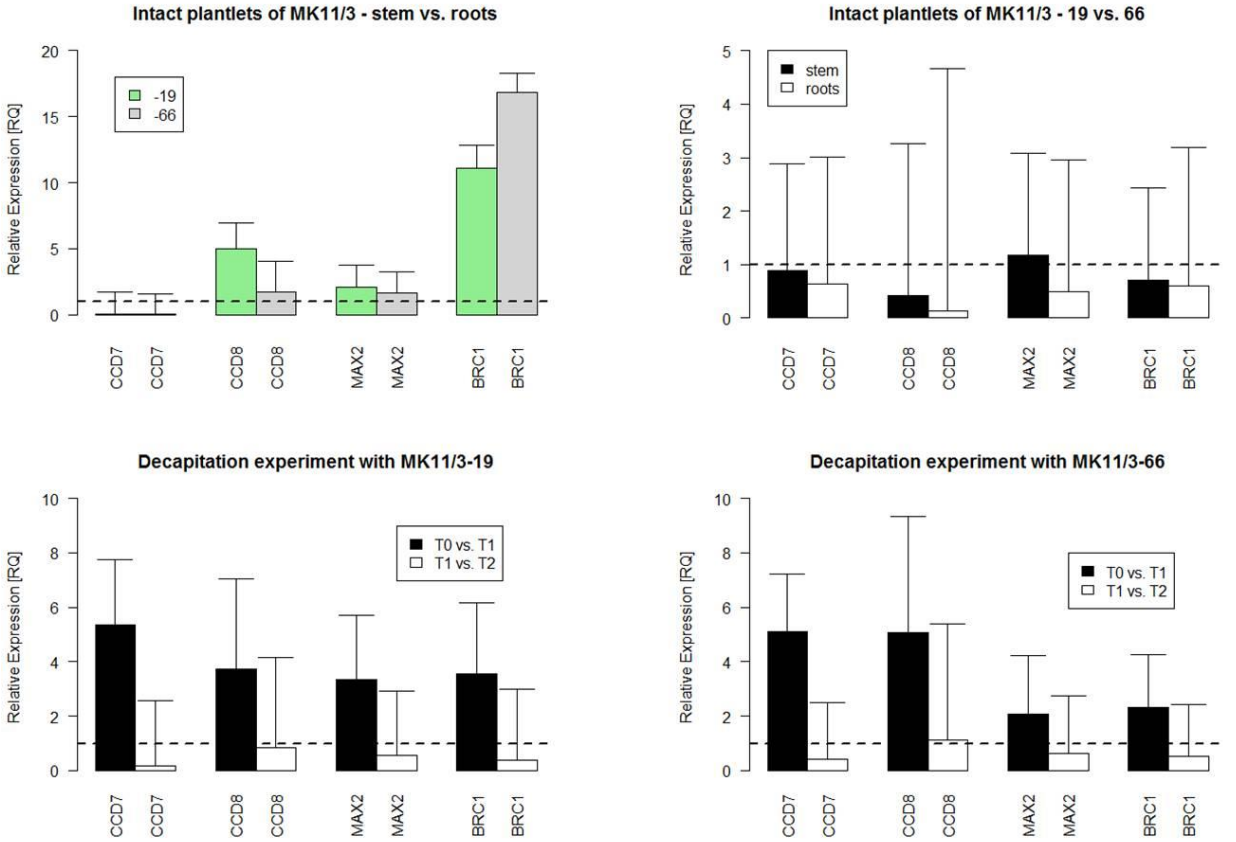


Figure S5.1: Relative Expression (RQ) of the candidate genes *CCD*7, *CCD*8, *MAX*2 and *BRC*1 under different conditions. The RQ was estimated with the ΔΔCq-method using the qpcrmix package (Gerhard *et al.*, 2013) of R software version 2.15.2 The dotted lines exemplifies an equal expression (RQ=1) between the two analysed states. The whiskers represent the standard error of the ΔΔCq estimates.

The contributions of the doctoral candidate to the manuscripts and publications

In the thesis "Genetic and genomic analysis of polyploid *Chrysanthemum* hybrids with emphasis on shoot branching" four manuscripts are presented.

In the first manuscript "The type of ploidy of chrysanthemum is not black or white: comparison of molecular to published cytological methods" the use of molecular markers is described to examine the inheritance of genetic loci and accordingly the type of ploidy of the ornamental chrysanthemum. The examination of this manuscript was conducted with a biparental F1 population, which was generated by me. Ina Menz and I performed the genotyping of this population using molecular markers. I analysed the molecular markers according to methods described by Dr. Stephan Schie in his dissertation "Charakterisierung der Genomstruktur polyploider Dahlien mithilfe molekularer Marker" from 2013. I have written the manuscript and it had been subsequently corrected by Dr. Stephan Schie, Dr. Marcus Linde and Prof. Dr. Thomas Debener. All presented tables and figures of this manuscript were created by me. The manuscript has been submitted to the peer-reviewed journal "Frontiers Plant Science" and is under review.

The second manuscript "Lack of structure in the gene pool of the highly polyploid ornamental chrysanthemum" analyses the genetic diversity and structure within germplasm of mainly European-cultivated chrysanthemums using AFLP® markers. The phenotyping of the plant material was conducted by Ina Menz and me. Data of the AFLP® markers were generated during the B. Sc. thesis of Ina Menz, which was supervised by me, Dr. Marcus Linde and Prof. Dr. Thomas Debener. I estimated measurements of the variability by different methods using these marker data and evaluated the minimum number of markers needed to represent the genetic distance between the genotypes accurately using bootstrap methods. I wrote the manuscript in cooperation with Ina Menz, which was subsequently corrected by Dr. Marcus Linde and Prof. Dr. Thomas Debener. All presented figures of the manuscript were designed by me. This work was published in the scientific, peer-reviewed journal "Molecular Breeding" (32: 339-348) in 2013.

In the third manuscript "Allelic variants of strigolactone pathway genes shape plant architecture: a case study on the inheritance of horticultural traits in chrysanthemum" marker-trait associations for important ornamental traits of chrysanthemum were identified by QTL mapping and association studies. Moreover, it describes to which extent DNA polymorphisms of candidate genes of the strigolactone pathway describe the phenotypic variation in shoot branching in natural chrysanthemum populations. This study used phenotypic data and genotypic data for the plant material of the manuscript of Chapter 2 and 3 and the molecular marker data were additionally extended by me for the association study. Furthermore, I phenotyped the investigated biparental population together with Ina Menz, the lab technician Jenny Rebentisch and the gardener Mario Esch. The candidate gene analysis was conducted by me. I have written the manuscript and designed the figures and tables. The manuscript had been corrected by Dr. Marcus Linde and Prof. Dr. Thomas Debener.

The fourth manuscript "Identification of a *CCD*7/*MAX*3 orthologue and the role of the strigolactone pathway for shoot branching in Chrysanthemum" compares different branching genotypes of the MK11/3 population reacting on treatments with the SL analogue CISA-1 and after decapitation under *in vitro* cultivation. The *in vitro* culture of these genotypes was established by collaborators of the company Hubert Brandkamp and was subsequently maintained and propagated by me. The CISA-1 analogue was kindly provided by Dr. Thomas Heugebaert. I conducted all the experiments *in vitro*, the qPCR analysis, the data mining and their statistical analysis. Dr. Marcus Linde and Thomas Debener were involved in planning and designing the experiments. I have written the manuscript and designed the figures and tables. The manuscript had been corrected by Dr. Marcus Linde and Prof. Dr. Thomas Debener.

Curriculum Vitae

Personal information

Name:	Maik Klie
Date/Place of birth:	10/01/1985 in Wernigerode
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University education

30/04/2014	Final examination and finish of the PhD studies.
09/2010 – 12/2013	PhD student at the Institute of Plant Genetics in the Department of Molecular Plant Breeding at the Leibniz Universität Hannover
10/2008 – 09/2010	Master of Science in Plant Biotechnology at the Leibniz Universität Hannover
10/2005 – 09/2008	Bachelor of Science in Plant Biotechnology at the Leibniz Universität Hannover

Employment history

01/2014 – till this date	Research assistant at the KWS Saat AG
09/2010 - 12/2013	Research assistant at the Institute of Plant Genetics in the Department of Molecular Plant Breeding at the Leibniz Universität Hannover - "Marker assisted selection for chrysanthemum to improve the plant architecture and flower size" - project funds from the Federal Ministry of Food, Agriculture and Consumer Protection (BMELV) via the Federal Office for Agriculture and Food (BLE) under the innovation support programme
08/2007 – 06/2010	Student assistant at the Institute of Plant Genetics in the Department of Molecular Plant Breeding at the Leibniz Universität Hannover
08/2006 – 09/2006	Work experience at Nordsaat Saatzucht GmbH in Böhnshausen
08/2004 – 04/2005	German national civilian service

Education

07/1995 – 06/2004	Secondary school in Ilsenburg
07/1991 – 06/1995	Primary school in Ilsenburg

Publications

Journal research papers and manuscripts

Klie M, Debener T (2011). Identification of superior reference genes for data normalisation of expression studies via quantitative PCR in hybrid roses (*Rosa hybrida*). *BMC Research Notes* 4: 518. 10.1186/1756-0500-4-518.

Klie M, Menz I, Linde M, Debener T (2013). Lack of structure in the gene pool of the highly polyploid ornamental chrysanthemum. *Molecular Breeding* 32: 339–348. 10.1007/s11032-013-9874-4.

Conference contributions

Lectures

Klie M (2011). Untersuchungen zur Diversität bei Schnittchrysanthemen. Tagung der Gesellschaft für Pflanzenzüchtung e. V. AG 18 (Zierpflanzen), Erfurt, DE.

Klie M (2012). Genetische Analyse morphologischer Merkmale bei Schnittchrysanthemen. WeGa PhD Tag der Doktoranden, Berlin, DE.

Klie M (2013). Marker associations for plant architecture in a polyploid ornamental: a case study on shoot branching in chrysanthemum. Plant and Animal Genome Conference XXI Section Ornamentals, San Diego, USA.

Klie M (2013). Genetische Analyse der Sprossverzweigung bei Schnittchrysanthemen. WeGa PhD Tag der Doktoranden, Straubing, DE.

Posters

Klie M, Linde M, Debener T (2012). Analysis of genetic variability in *Chrysanthemum indicum* germplasm for genes involved in the control of shoot branching. Plant and Animal Genome Conference XX, San Diego, USA.

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Nach Goethe *"lässt sich eine wahrhafte Dankbarkeit mit Worten [leider] nicht ausdrücken"*, dennoch möchte ich diese Stelle nutzen um einigen Menschen zu danken.

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