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Inheritance of *Barley yellow dwarf virus* resistance in maize

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Abbreviations

BYD	Barley yellow dwarf
BYDV	*Barley yellow dwarf virus*
cM	centiMorgan
DAS-ELISA	double-antibody sandwich enzyme-linked immunosorbent assay
EH	ear height
EX	virus extinction
FT	flowering time
GBS	genotyping by sequencing
GWAS	genome wide association study
H^2	heritability
HR	hyper sensitive reaction
INDEL	insertions and deletions
IR	infection rate
LD	linkage disequilibrium
MAS	marker assisted selection
MCDV	*Maize chlorotic dwarf virus*
MDMV	*Maize dwarf mosaic virus*
NGS	next generation sequencing
PH	plant height
RAD	restriction site-associated genomic DNA
RE	red edges
SNP	single nucleotide polymorphism
SCMV	*Sugarcane mosaic virus*
QTL	quantitative trait locus
WSMV	*Wheat strike mosaic virus*

1. General Introduction

Barley yellow dwarf virus

Barley yellow dwarf (BYD) is the most widespread virus disease of small grain cereals (Plumb, 1983) and was first detected in barley by Oswald and Houston (1951). It is caused by the BYD virus (BYDV), a luteovirus belonging to the family *Luteoviridae* (Miller et al., 2004) which is phloem-restricted (Lister and Rochow, 1979) and transmitted by different aphid species (Oswald and Houston, 1951).

Rhopalosiphum padi, one of the main cereal aphids, is deemed to be a good vector for BYDV. It has three main flight periods. In autumn, the aphids migrate to autumn-sown cereals and transmit BYDV from different infection sources, such as volunteer cereal plants, grasses, and also from maize to winter wheat and winter barley. Until winter, the aphids can distribute the virus in the field. Later in autumn, *R. padi* migrates to its winter host, the bird-cherry tree, for the sexual reproduction and overwintering. In spring, the virus-free *R. padi* migrates from the winter host to the winter cereals, where the aphids acquire the virus. During the flight in early summer, the aphids further transmit the virus in the autumn sown cereal fields as well as to spring cereals and maize. The most important virus epidemiology period is the alteration of the aphid vectors from the ripening winter and spring cereals to maize and perennial grasses, before in early autumn the aphids colonize the new sown winter cereals (Henry and Dedryver, 1989).

The importance of maize as host plant in the epidemiology of BYDV suggests in turn a relationship between the BYDV occurrence in maize and the infection rate of winter cereals (Plumb, 1983).

BYDV in maize

With climate change, winters are becoming milder in temperate climate zones which enables aphids to overwinter in cereal crops (Irwin, 1990). This leads to a continuous spread of the virus during autumn and winter in the field and to an earlier presence of larger aphid populations in spring time. In addition, high temperatures in spring lead to an early invasion of the vectors from the winter cereals to maize, and therefore to an early attack of maize plants (Harrington et al., 2007). As previous studies revealed that crop plants are especially sensitive to BYDV infection in early developmental stages (Haack et al., 1999), this suggests an increasing impact of BYD on all cereals and especially maize in the future. The symptoms detected in BYDV infected maize are red bands at the edge of the leaves and interveinal yellowing of leaves (Loi et al., 2004). Furthermore, the results of earlier studies suggested that BYDV infection in maize might lead to a reduction of plant height (Beuve et al., 1999; Loi et al., 2004), total plant fresh weight (Panayotou, 1977), and grain yield (Beuve et al., 1999; Pearson and Robb, 1984). However, a systematic analysis of the influence of BYDV infection on plant performance trait on a diverse set of genetic material is still missing.
The aphid transmitted BYDV can be controlled by spraying insecticides. However, this is a cost and labor intense approach and harmful for the environment. Therefore, breeding for BYDV resistance is the best alternative to control the disease and avoid reduction of plant performance caused by the virus (Ordon et al., 2004).
Not all infected genotypes show symptoms (Grüntzig et al., 1997) and, thus a distinction can be made between tolerant and resistant genotypes. Tolerant genotypes are symptomless or show only week symptoms but allow BYDV to

replicate. Resistant genotypes, in contrast, do not show symptoms and the virus can not or only to a low extent replicate in the plant (Osler et al., 1985). Only with resistant maize it is possible to break the epidemiological cycle of BYDV and also to improve the BYDV situation in other cereals. The prerequisite for improving the BYDV resistance by breeding is genetic variation for the trait of interest. Loi et al. (1986) described the maize inbred Ky226 as tolerant, because it did not show symptoms. In contrast, the maize inbred W64A was described to be highly susceptible. In experiments of Grüntzig and Fuchs (2000), FAP1360A showed a very low infection rate and low extinction values in enzyme-linked immunosorbent assay (ELISA) and also D408 was resistant. Furthermore, it was shown that FAP1360A is resistant against *Sugarcane mosaic virus* (SCMV) (Duß le et al., 2000). My study was based on segregating populations derived from crosses of these maize inbreds.

Identification of molecular markers for BYDV resistance in maize

Previous studies described genetic material showing not only tolerance but also resistance to BYDV (Grüntzig and Fuchs, 2000; Loi et al., 1986). Different resistance mechanisms against viruses in plants have been reported. Many plant species defend themselves passively by strengthened cell walls (Goldbach et al., 2003). Other mechanisms are active defense mechanisms, most commonly the hypersensitive response (HR). Lamb and Dixon (1997) reported that a fast production of oxidants is a typical indicator for the HR. For *Sugar cane mosaic virus*, two major resistance genes have been mapped to chromosomes 6 (*Scmv1*) and 3 (*Scmv2*) (Melchinger et al., 1998) as well as three minor genes to chromosome 10 (Xia et al., 1999; Zhang et al., 2003). In SCMV resistant maize plants, the virus spread was slower than in susceptible plants which leads to the assumption that in resistant plants the virus spread through the leaf vascular system is inhibited (Quint, 2003). Zambrano et al.

(2014) identified furthermore quantitative trait loci (QTL) on the chromosomes 1, 2, 3, 6 and 10 contributing to resistance against six virus diseases. Furthermore, Jones et al. (2011) and McMullen and Simcox (1995) identified the locus *wsm3* on chromosome 10 which explains variation of *Wheat strike mosaic virus* (WSMV) resistance. Jones et al. (2004) described the locus *mcd1* to contribute to resistance against *Maize chlorotic dwarf virus* (MCDV).

But, to the best of my knowledge, nothing is known about the mechanisms and the inheritance of BYDV resistance and tolerance in maize. Furthermore, there are no BYDV resistance genes described in maize yet. In this study I addressed the questions, which genome regions are involved in the BYDV resistance in maize.

A promising approach to identify markers genetically linked to the trait of interest is linkage mapping using biparental populations. Another approach is a genome wide association study (GWAS), which uses a diverse germplasm set having the advantage that a large number of alleles per locus can be surveyed simultaneously, compared to only two alleles in a biparental cross. Furthermore, because of historical recombinations in GWAS populations, the mapping resolution is higher compared to classical linkage mapping (Flint-Garcia et al., 2005).

However, the disadvantage of GWAS is that alleles with a low allele frequency can remain undetected. Due to a balanced allele frequency in segregating populations, classical linkage mapping has the advantage of higher QTL detection power compared to GWAS (Würschum, 2012). An improved method of linkage mapping with biparental populations is the use of connected populations which share parental inbred lines (Bardol et al., 2013). In such connected populations, it is more likely to find alleles of interest because in contrast to a single biparental population more alleles over multiple genetic backgrounds can be considered (Bardol et al., 2013). This in turn increases the probability that a QTL will be polymorphic in at least one population (Blanc et al., 2006).

Objectives

The goal of my thesis research was to contribute to unravel the inheritance of BYDV resistance in maize. The main objective was to combine the high detection power of linkage mapping and the high resolution of a genome wide association mapping for the identification of molecular markers to understand the inheritance of BYDV resistance in maize and to enable a marker assisted selection in breeding of BYDV resistant maize.

In particular, the objectives were to

1. determine phenotypic and genotypic variation in five segregating populations of maize with respect to BYDV tolerance and resistance;
2. determine genetic variation with respect to BYDV resistance in a broad germplasm set of maize;
3. quantify the influence of BYDV infection on the plant traits plant height, ear height, and flowering time;
4. identify genome regions which are involved in the BYDV resistance mechanism by a GWAS;
5. and to validate the genome regions with a linkage mapping approach in five connected biparental crosses.

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Natural variation for BYDV resistance in maize

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Abstract

With increasing winter temperatures due to climate change, Barley yellow dwarf virus (BYDV) is expected to become a prominent problem also in maize cultivation. Breeding for resistance is the best alternative to control the disease and break the transmission cycle of the virus. The objectives of our study were to (I) determine phenotypic and genotypic variation in five segregating populations of maize with respect to BYDV tolerance or resistance as well as (II) quantify the influence of BYDV infection on plant performance traits. In 2011, five segregating populations with a total of 445 genotypes were grown at two locations in Germany. Plants were inoculated with BYDV-PAV transmitted by aphids of the species *Rhopalosiphum padi*. We observed considerable genotypic variance for the traits virus concentration as measured by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) as well as expression of symptoms. Furthermore, heritabilities were high for the plant performance traits ear height and plant height. Correlation coefficients between all pairs of traits were significantly different from 0 ($P < 0.05$). Genotypes of the inoculated variant were reduced in plant height by 3 cm, ear height by 6 cm, and flowered 3 days earlier compared to genotypes of the non-inoculated variant. The results of our study suggested a high potential for breeding of BVDY resistant / tolerant maize.

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Genes involved in barley yellow dwarf virus resistance of maize

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Abstract

With increasing winter temperatures in Europe due to climate change, *Barley yellow dwarf virus* (BYDV) is expected to become a prominent problem in maize cultivation. Breeding for resistance is the best strategy to control the disease and break the transmission cycle of the virus. The objectives of our study were (i) to determine genetic variation with respect to BYDV resistance in a broad germplasm set and (ii) to identify single nucleotide polymorphism (SNP) markers linked to genes that are involved in BYDV resistance.

An association mapping population with 267 genotypes representing the world's maize gene pool was grown in the greenhouse. Plants were inoculated with BYDV-PAV using viruliferous *Rhopalosiphum padi*.

In the association mapping population, we observed considerable genotypic variance for the trait virus extinction as measured by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) and the infection rate. In a genome wide association study, we observed three SNPs significantly (false discovery rate (FDR)=0.05) associated with the virus extinction on chromosome 10 explaining together 25% of the phenotypic variance and five SNPs for the infection rate on chromosomes 4 and 10 explaining together 33% of the phenotypic variance.

The SNPs significantly associated with BYDV resistance can be used in marker assisted selection and will accelerate the breeding process for the development of BYDV resistant maize genotypes. Furthermore, these SNPs were located within genes which were in other organisms described to play a role in general resistance mechanisms. This suggests that these genes contribute to variation of BYDV resistance in maize.

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Linkage mapping of Barley yellow dwarf virus resistance in connected populations of maize

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Abstract

With increasing winter temperatures due to climate change, *Barley yellow dwarf virus* (BYDV) is expected to become an increasing problem in maize cultivation in Germany. Earlier studies revealed that BYDV has a negative impact on maize performance. Molecular markers would accelerate the development of BYDV resistant maize. Therefore, the objectives of this study were (i) the identification of quantitative trait loci (QTL) for BYDV resistance in five connected segregating maize populations in a field experiment and (ii) their comparison with the QTL detected under greenhouse conditions.

In linkage analyses of the traits virus extinction, infection rate, and the symptom red edges, a highly associated major QTL was identified on chromosome 10. This QTL explained 45% of the phenotypic variance for the traits virus extinction and infection rate and 30% for the symptom red edges. We could show that BYDV resistance traits are oligogenically inherited. The QTL on chromosome 10 could be observed in the connected linkage analyses and in the single population analyses. Furthermore, this QTL could also be confirmed in the greenhouse experiment. Our results let suggest that this QTL is involved in multiple virus resistance and the markers are promising for marker assisted selection.

5. General Discussion

Climate change and its impact on plant diseases, especially BYDV

Climate change models suggests that an increase of the global average temperature of up to 6°C is expected by the year 2100 (Jones, 2009). Furthermore, it is predicted that the temperature is raising faster in northern regions compared to regions near the equator. In the last years, changing climatic conditions and their influence on plant pathogen distribution has been studied. Results of these studies indicate that climate change can modify stages and rate of development of the pathogens. Aphids have a short generation time and low developmental threshold temperatures. Therefore, they are expected to respond extremely quick to climatic changes with an increased reproduction (Harrington et al., 2007). Already a 3°C warming leads to an increase of seven generations per year (Yamamura and Kiritani, 1998).

It was observed that climate change leads to a shift of pathogens and hosts in their geographical distribution (Coakley et al., 1999). Due to mild winters, Nematoda and viruses move towards the equator (Bebber et al., 2013). In contrast many plant diseases and pests move polewards because there they find favorable conditions (Bebber et al., 2013). For example, on the northern hemisphere Acari, Bacteria, Fungi, Oomycota and insects e.g. Coleoptera, Diptera, Hemiptera, Isoptera, and Lepidoptera were increasingly

observed towards the North since 1960. It is also expected that insects, such as BYDV transmitting aphids become an increasing problem in northern regions because they survive in larger populations because of milder winter temperatures. Due to this conditions the flying period of aphids was shifted one month earlier in spring in the last years (Gregory et al., 2009). An infection of maize in early developmental stages increases the damage of the plant. This is because the organ development in plants is not completed and they stay reduced in their development (Huth, 1994). Due to these changes resulting from climatic shift it is expected that BYDV is becoming an increasing problem in maize cultivation in Germany.

Impact of BYDV on plant performance and yield

The reaction of maize to BYDV infection was first studied in the Southern European countries Spain (Comas et al., 1993), Italy (Coceano and Peressini, 1989; Loi et al., 1986) and France (Beuve et al., 1999; Haak et al., 1999). Grüntzig et al. (1997) and Grüntzig and Fuchs (2000) studied the occurrence and influence of BYDV in maize in Germany.
BYDV occurs with different frequencies in almost all maize fields (Huth, 1994). An infection of maize by BYDV can be recognized by two major leaf symptoms. Infected plants show intervenial yellow stripes on the leaves (YS) or red bands at the edge of the leaves (RE) (Beuve et al., 1999; Grüntzig et al., 1997; Grüntzig and Fuchs, 2000; Loi et al., 2004). The occurrence of symptoms depends on the reaction of the maize genotype to BYDV infection, the developmental stage at which infection appeared, and the environmental influences (Huth, 1994). In the frame of my thesis work, YS and RE were scored. I observed that the symptom RE showed a higher heritability compared to the symptom YS. The reason is that YS are stronger influenced by the environment than RE. Environmental influences are probably the reason

why YS were also observed in the non-inoculated control. Environmental influences leading to YS could be e.g. nutrient deficiencies/excess (Marschner, 1995). Furthermore, iron inefficient maize plants cannot take up adequate amounts of iron from the soil which leads to chlorosis and YS on the leaves (Curie et al., 2001). Chlorophyll deficiency (Beadle, 2014) and different virus diseases like leafhopper-transmitted *Maize yellow stripe virus* (Ammar et al., 1990) can also cause YS. Such influences may lead to a high error variance resulting in a low heritability for YS in my study although the plants were inoculated artificially. For the symptom RE, in contrast, the genotypic variance was higher than the error variance leading to a high heritability. This indicated that RE is the more reliable BYDV symptom and therefore, it was mainly used for further analysis in my thesis work.

The results of previous studies suggested that BYDV infection has a negative impact on plant performance and leads in maize to a reduction of plant height (Beuve et al., 1999; Loi et al., 2004), total plant fresh weight (Panayotou, 1977), and grain yield (Beuve et al., 1999; Pearson and Robb, 1984). In my study, plant height was reduced on average by three cm and ear height by six cm in inoculated plants compared to non-inoculated plants. Furthermore, flowering time was three days earlier in BYDV infected plants compared to non-inoculated plants. The reductions were not in all populations significant ($\alpha = 0.05$). However, susceptible plants with EX ≥ 0.5 were stronger reduced compared to resistant plants with EX <0.5.

I observed no significant ($\alpha = 0.05$) differences in yield and quality parameters between inoculated and non-inoculated plants. A reason for this finding could be that due to practical reasons only a small sample size of four plants per genotype and treatment and also only the five parental inbreds were examined. Yield has a high genetic complexity because it is influenced by many small effect genes. Furthermore, yield is influenced by environmental factors making the evaluation even more difficult (Sibov et al., 2003). This could have led to a low statistical power to detect significant yield and quality reductions. To improve this, a higher number of observations would be required.

Control of BYDV in maize by breeding resistant genetic material

Maize and other cereals are the most cultivated crops in the world. In Germany, the cultivation area of maize is increasing, mainly for biogas production (Gevers et al., 2011). Most crop rotations are very narrow between small grain cereals and maize which are all known to be host plants for BYDV. In such maize-cereal-maize crop sequences, maize serves as an important summer host for BYDV. The virus can replicate in the maize plant during summer which bridges the time between the cereals are harvested and sown again and therefore complete the transmission cycle of BYDV.
To avoid the increasing damage by BYDV on maize and other cereals with increasing cultivation and climatic changes, as outlined above it is important to break the transmission cycle and find a sustainable solution to control the disease in maize. The virus itself can not be controlled directly by any type of chemical treatment, only the aphids can be controlled by insecticide spraying. This method is often prophylactic and therefore expansive and harmful for the environment (Ordon et al., 2004). Furthermore, the application of insecticides to aphids in maize is not allowed in Germany. Therefore, the development of resistant maize is the only possibility to control the virus.
The phenotypic selection of BYDV resistant maize is difficult because it was observed that some genotypes are not resistant to BYDV but only tolerant (Osler et al., 1985). That means they do not show symptoms, but the virus can replicate in the plant. To be able to detect resistant genotypes, the virus content has to be measured by double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA). But the inoculation via aphids and the measurement of BYDV content is labor-, cost-, and time consuming and therefore difficult to be included in practical breeding programs. To accelerate the selection of resistant lines in the breeding process, molecular markers linked to BYDV resistance are an important avail. With the knowledge of molecular markers for BYDV resistance in maize, it is possible to develop BYDV resistant cultivars by marker assisted selection (MAS). With

BYDV resistant maize cultivars the damage in maize can be reduced. Furthermore, the situation in other cereals can also be improved because BYDV can not replicate in resistant maize and therefore, the infection pressure on the following winter cereals is reduced.

Phenotypic and genotypic variation of BYDV resistance in diverse genetic material

A genotypic variance with regard to BYDV resistance traits is required to achieve breeding progress. Furthermore, genotypic variance leads to a high statistical power for the detection of genome regions contributing to variation of BYDV resistance. To reach a broad variation within the populations for the linkage mapping experiments in the development of the populations it was important to include parents that carry resistance and susceptibility alleles. The parental inbreds of the segregating populations were chosen based on the information of their BYDV resistance/susceptibility from the literature. Loi et al. (1986) described Ky226 as tolerant whereas W64A was described to be susceptible. Experiments of Grüntzig and Fuchs (2000) showed that FAP1360A and D408 were resistant. Furthermore, it was shown that FAP1360A is also resistant against SCMV (Duß le et al., 2000). To reach a dissection in the populations, such inbreds were crossed that differed in their BYDV resistance. Only population C was a crossing between two resistant inbreds in order to examine, if the two inbreds carry the same resistance allele and showed the lowest variation. However, in all other populations a broad variation with regard to BYDV resistance was observed with the exception of population C.

The association mapping population used in my study (Flint-Garcia et al., 2005) showed an even broader variation than the linkage mapping populations. An explanation is that this population represents the diversity of maize breeding material from all over the world. In such genetically diverse populations, a high recombination took place over a long time of selection

processes. This leads to a higher polymorphy level. This genetic diversity results in a continuous phenotypic variation which could be observed also in my study for the traits virus extinction (EX) and infection rate (IR). The traits EX and IR were continuously distributed across the whole population and also the subgroups showed a continuously distribution.
The continuous variation indicates that BYDV resistance can be influenced by the environment. However, the high heritabilities (H^2) suggested that this might be rather due to an oligogenic inheritance. The broad variation in the segregating populations as well as the association mapping population provides a good basis for the identification of trait-genotype associations.
The traits EX and IR did not cluster in the subgroups and are not strongly influenced by population structure. This could be shown by the phenotypic variation explained by the population structure which was only 7.0% for the trait EX and 4.7% for the trait IR. Therefore, the whole population was analysed but not each single population.

Association and linkage mapping for the identification of molecular markers linked to BYDV resistance in maize and possible resistance mechanisms of the identified genes

Due to a higher recombination rate in an association mapping population compared to linkage mapping populations the mapping resolution in a GWAS is increased compared to classical linkage mapping. Another advantage of an association mapping population is the large number of alleles per locus that can be studied simultaneously (Flint-Garcia et al., 2005). GWAS, however, has the disadvantage that if population structure is not considered, spurious associations can occur (Pritchard et al., 2000). Therefore, population structure was estimated for the association mapping population by

Flint-Garcia et al. (2005), using 89 SSR markers with the software STRUCTURE (Pritchard et al., 2000) and was included in the model. Furthermore, a kinship matrix (K) was included to define the degree of genetic covariance among individuals (Yu and Buckler, 2006) and integrated both in the mixed model for the genome wide association study.

In the GWAS, three single nucleotide polymorphisms (SNPs), significantly (FDR=0.05) associated with EX, were identified on chromosome 10 explaining in a simultaneous fit 25% of the phenotypic variance. All three SNPs were located in the gene GRMZM2G018027. This gene is not described in maize yet but the best hit in *Arabidopsis thaliana* is the gene OXS3 which was described to be expressed during oxidative stress reaction. This gene could induce a reaction leading to callose deposition at the plasmodesmata of plant cells, reducing virus spread in the plant (Wang and Culver, 2012). Furthermore, the SNPs significantly associated with IR on chromosome 10 and 4 were located in gene regions, which were in other plants described to be involved in resistance mechanisms. This suggests that genes involved in general resistance mechanisms are also involved in BYDV resistance in maize.

The statistical method used in my GWAS considers genetic and phylogenetic relationship by taking the population structure and a kinship matrix into account. Therefore, the problem of detecting false-positive SNPs is reduced. However, false-positives can still occur and in principle a validation by using another method or environment is required. Therefore, in my study I carried out a connected linkage analysis using five connected segregating populations in order to validate the results of the GWAS. With this approach the advantages of both methods were combined that are the high detection power of the linkage mapping as well as the high mapping resolution and the high number of alleles of the GWAS. Moreover, it was possible to compare the results of both methods in different plant material.

The significantly associated markers on chromosome 10, identified by the GWAS, were validated in the connected linkage analysis. The confidence interval of the QTL identified in the linkage analysis, explaining 45% of the phenotypic variance for the traits EX and IR colocalized with the SNPs iden-

tified by the GWAS on chromosome 10. Both GWAS and linkage mapping showed that BYDV is oligogenically inherited because one major peak explained a high proportion of the phenotypic variance. The genome region on chromosome 10 identified in my study were already described to be involved in the resistance to *Maize dwarf mosaic virus* (MDMV) (Zambrano et al., 2014), WSMV (Jones et al., 2011), MCDV (Jones et al., 2004) and for SCMV (Xia et al., 1999; Zhang et al., 2003). This shows that the genome region on chromosome 10 could be involved in resistance mechanism to multiple viruses resistance. However, this requires further research.
In an analysis of the individual segregating populations, the QTL region on chromosome 10 was colocalized in all of the populations, except population C which is a cross between two resistant parental inbreds. The reason is that the two resistant parental inbreds of population C carry the same resistance gene and did not segregate in this region. Furthermore, also the main QTL locations for EX and IR from the greenhouse and the field experiment colocalized.

Genotype-environment interaction - a comparison between the field and the greenhouse experiments

To be able to compare the H^2 observed for the five segregating populations in the field experiments to that observed in the greenhouse experiments, H_j^{*2} was calculated on a plot basis (Smalley et al., 2004). H_j^{*2} were almost the same in the greenhouse and the field. This shows that the environmental influence was comparable in the field and the greenhouse.
I observed that the traits assessed in the field and the greenhouse correlated significantly (α=0.01). However, the correlation coefficients were with 0.43 and 0.44 not perfectly. This can be explained by the genotype-environment interaction which can result from differences for example in light conditions,

temperature, humidity, soil nutrients, water application and plant density in the greenhouse compared to the field. Genotype-environment interaction can give rise to problems for an indirect selection when plants are selected in the greenhouse which will in practice be grown under field conditions. Especially in population B and C the genotype-environment interaction led to higher EX and IR values in the greenhouse compared to the field.

Genotype-environment interaction can also lead to the detection of different QTL (Brachi et al., 2010) if the environmental conditions differ in greenhouse and field experiments. In the linkage analysis of the data examined in the greenhouse experiment, some additional QTL were detected compared to the field experiments. One additional QTL for the trait EX on chromosome 1 was identified in population B. Meihls et al. (2013) identified a benzoxazionoid QTL in maize in this region which causes aphid resistance and is associated with low levels of 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one glucoside methyltransferase leading to an increased aphid resistance by promoting callose deposition. That means it is possible that in at least this populations BYDV resistance could be supported by a resistance against the aphid. If the aphids can not suck the sap of the plants, no virus can be transmitted.

Another additional QTL was detected on chromosome 5 in population C. For this QTL on chromosome 5 at the position 60.8cM, a significant (α=0.01) epistatic interaction was detected with the position at 80.7cM on chromosome 6. Because this epistatic interaction explains 21% of the phenotypic variance it contributes an important part to BYDV resistance, at least in this population. However, the QTL from the field analysis on chromosome 10 for EX and IR could be validated in the greenhouse experiment in the connected analysis as well as in all populations except in population C.

The use of QTL and markers for BYDV resistance in practical breeding

For practical breeding the results from field experiments are very useful for MAS. Nevertheless, in each environment genotype-environment interaction can lead to different results. Therefore, the additional QTL identified in the greenhouse can also be of interest for MAS. The markers identified for BYDV resistance in this study are promising for the use in MAS because a few markers explain a high proportion of the phenotypic variance of the BYDV resistance traits. The narrow confidence intervals for EX and IR show that the recombination rate is low within the flanking markers and therefore, the markers are closely linked to the gene conferring resistance to BYDV.

Genotyping by sequencing – Advantages and challenges of a promising approach for the future

Instead of genotyping single SNPs, nowadays it is possible to sequence complete parts of the genome. Genotyping by sequencing (GBS) provides a high number of molecular markers because many parts of the genome are sequenced (Baird et al., 2008). Restriction site-associated genomic DNA (RAD) marker is a GBS approach, where DNA is digested by a restriction enzyme, DNA fragments are then sequenced and the reads are aligned to the reference sequence. Then SNPs as well as insertions and deletions (INDELs) can be detected by aligning the reads to the reference sequence. This method was first described by Baird et al. (2008) in fish and later successfully conducted in different crop species like rape seed, maize and barley (Bus et al., 2012; Elshire et al., 2011; Mascher et al., 2013).
There are, however, some challenges of this method which will be discussed

in the following. In next generation sequencing (NGS), sequencing errors can occur due to the inherent bias of polymerase chain reaction in data preparation or due to genome sequences, e.g. under-represented GC rich regions or AT-rich repetitive sequences (Nakamura et al., 2011). With a quality check for the quality score of the reads, sequencing errors can be reduced but nevertheless, they can still lead to false SNP calls.

Repetitive sequences and sequencing errors can further lead to alignment problems. To identify SNPs in my study, an alignment with up to three missmatches was allowed. The coverage can become very low if only few missmatches are allowed and even more if there are sequencing errors (Nakamura et al., 2011). Nevertheless, the more miss-matches are allowed, the higher is the risk of false alignment. If the restriction enzyme cuts in a repetitive sequence, the read will later fit at too many regions and can be falsely aligned. To reach a coverage of three reads per alignment, in my study I selected the restriction enzyme *Kpn1* based on their fragment cuts in silico. The disadvantage of this restriction enzyme, however, is that it is not sensitive to methylation and therefore, it is not unlikely to cut at repetitive regions. This could be a further explanation for false alignment of some reads in my study leading to difficulties in the analysis (Elshire et al., 2011).

Sequencing methods are still expensive, even though the prices are decreasing. To reduce costs it is possible to multiplex 96 genotypes per lane. The disadvantage of high multiplexing, however, is that a lower number of reads per alignment is present. Furthermore, due to missing data from false alignment and sequencing errors there are gaps at different positions. That means not all individuals have a genotyic marker information at the same positions. To fill such gaps haplotype blocks can be filled by imputing (Elshire et al., 2011). However, I observed an unbalanced allele frequency in the populations which deviate from the expected 1:1 ratio in biparental populations. This shows that there are still some difficulties in GBS methods.

Due to this difficulties it was not possible to create a genetic map fitting to the physical position of the markers. To use markers for linkage analysis a genetic map is required and therefore, I decided to create the map based on markers from a MaizeSNP50 array (Ganal et al., 2011) for the linkage

mapping analysis.

Conclusion and Outlook

In the GWAS carried out in my study, I identified a gene on chromosome 10 which is highly associated with BYDV resistance in maize. The three SNPs on chromosome 10 explained together 25% of the phenotypic variance for EX, showing that BYDV reistance is an oligogenically inherited trait. I was able to validate this genome region furthermore by linkage mapping with five connected biparental populations, where the significantly associated QTL for EX explained 45% of the phenotypic variance. This illustrated the potential of the resistance gene to be broadly applicable. With only a few markers, explaining a high proportion of the phenotypic variance it is promising that MAS based on this markers leads to an improvement of BYDV resistance in maize. The GWAS resulted in a candidate for the BYDV resistance on chromosome 10. This gene was validated by a mutant approach. However, this should be complemented by fine mapping in segregating populations.
Besides quantitative genetics further research could also be investigated in understanding resistance mechanisms. This can be done by e.g. comparative genomics which compares genomic structures, like sequences and genes in different organisms. Maize can be compared with such approaches like synteny of resistance mechanisms of other plants. Furthermore, based on the information of this study, mutant screening approaches could be further expanded with homozygote mutants and in a larger scale with more plants to be able to reduce the error and enables a comparison by a real-time-quantitative polymerase chain reaction (qRT-PCR) expression test. In another approach the gene can be over expressed or knocked out, to see if the gene is essential for BYDV resistance. To further follow the hypothesis that callose deposition stops virus spread in the plant a coloring approach of callose with aniline blue could further be of interest.
For breeders and farmers it would be also of interest how strong yield quality

is really reduced by BYDV. To clarify this, more plants per plot need to be evaluated in the field. As it is expected that BYDV becomes an increasing problem in maize cultivation in Germany, my goal was to provide breeders molecular markers for MAS. This study will help to improve the development of resistant maize cultivars and is a good basis for further scientific research.

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6. Summary

Barley yellow dwarf (BYD) is one of the economically most important virus diseases in cereals. Due to increasing winter temperatures it is expected that BYD will become an increasing problem in maize cultivation. In earlier studies, it was reported that BYD has a negative impact on plant performance of maize. BYD virus (BYDV) is transmitted by aphids and the best control of the virus is the development of resistant maize cultivars. Therefore, the first objectives of my thesis research were to (i) determine phenotypic and genotypic variation in five segregating populations and in a broad germplasm set of maize with respect to BYDV tolerance and resistance as well as to (ii) quantify the influence of BYDV infection on the plant traits plant height, ear height, and flowering time.
I observed a negative impact of BYDV infection on maize plant traits which shows that the development of resistant maize cultivars is of high importance for maize cultivation. Furthermore, in the connected biparental populations as well as in the association mapping population, I observed a high genotypic variance with regard to BYDV resistance which is the requirement for successful breeding and the identification of genome regions which contribute to BYDV resistance.

The evaluation of BYDV resistance by the inoculation with BYDV and by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) is difficult to be included in the breeding process. Therefore, molecular markers are of high importance for the improvement of BYDV resistance

by breeding. Therefore, the objective of this study was the (iii) identification of genome regions which are involved in the BYDV resistance by a genome wide association study (GWAS).
For the BYDV resistance traits, significantly (α=0.01) associated SNPs were identified in the GWAS on chromosome 10 and 4. The SNPs identified for virus extinction on chromosome 10 explained in a simultaneous fit 25% of the phenotypic variance and were located in gene regions which were in other plants described to be involved in resistance mechanisms. This suggests that BYDV resistance is inherited oligogenically and that genes involved in general resistance mechanisms are also involved in BYDV resistance in maize.

GWAS has the advantage that a large number of alleles per locus can be surveyed simultaneously, and because historical recombinations can be used, the mapping resolution is higher compared to classical linkage mapping. Nevertheless, genes contributing to phenotypic variation which show a low allele frequency can remain undetected. Due to a balanced allele frequency in segregating populations, linkage mapping has the advantage of higher QTL detection power compared to GWAS. Therefore, the objective of this study was to (iv) validate the genome regions with a linkage analysis in connected biparental crosses.
The genome region on chromosome 10 which was identified in the GWAS to be linked to BYDV resistance could be validated in the linkage mapping study with connected populations as well as in the single populations. Furthermore, the QTL on chromosome 10 colocalized with the QTL identified in controlled greenhouse conditions. In earlier studies, QTL for other virus resistances were identified on chromosome 10. This suggests that these genes are involved in multiple virus resistances. The identified genome regions explain 45% of the phenotypic variance and are, therefore, promising for the use in MAS.

The broad genotypic variation with regard to BYDV resistance, observed in my thesis research, provided a good basis for the successful identification of molecular markers which are associated with BYDV resistance in maize. The markers identified in my study by GWAS were validated by a linkage

mapping approach and are promising for the use in marker assisted selection on BYDV resistance in maize breeding.

7. Zusammenfassung

Die Gerstengelbverzwergung (Barley yellow dwarf, BYD) gehört wirtschaftlich zu den wichtigsten Viruskrankheiten im Getreide. Aufgrund steigender Wintertemperaturen wird auch in Mais erwartet, dass die BYD in Zukunft ein wachsendes Problem wird, zumal aus bisherigen Studien bekannt ist, dass die BYD einen negativen Einfluss auf Mais hat. Die effektivste Methode zur Bekämpfung des BYD Virus (BYDV), welches von Aphiden übertragen wird, ist die Züchtung von resistenten Maissorten. Deshalb waren die ersten Ziele meiner Doktorarbeit (i) die Erfassung der genotypischen Variation für die BYDV-Resistenz in fünf spaltenden Populationen und in einem diversen Mais-Set, sowie (ii) die Beobachtung des Einflusses der BYDV-Infektion auf die Pflanzenmerkmale, Pflanzenhöhe, Kolbenhöhe und den Blühzeitpunkt.
In der aktuellen Studie wurde beobachtet, dass eine BYDV-Infektion einen negativen Einfluss auf Pflanzenmerkmale von Mais hat - was die Relevanz einer Verbesserung der BYDV-Resistenz hervorhebt. Des Weiteren wurde sowohl in den verbundenen spaltenden Populationen als auch in der Assoziationskartierungspopulation eine hohe genotypische Variation für die BYDV-Resistenz beobachtet. Diese stellt eine wichtige Voraussetzung für eine erfolgreiche Assoziationskartierung dar.

Die Evaluierung hinsichtlich der BYDV-Resistenz ist schwer in den Züchtungsprozess zu integrieren, da die Virus-Inokulation mit Blattläusen und die Bestimmung des Virusgehalts durch DAS-ELISA sehr aufwändig

sind. Für die züchterische Verbesserung der BYDV-Resistenz sind molekulare Marker von großer Bedeutung. Deshalb war ein weiteres Ziel der Arbeit (iii) in einer genomweiten Assoziationskartierung (GWAS) Genomregionen zu identifizieren, die an der BYDV-Resistenz in Mais beteiligt sind.
In der GWAS wurden signifikante (α=0.01) SNPs auf Chromosom 4 und 10 für die BYDV-Resistenzmerkmale identifiziert. Die SNPs die auf Chromosom 10 für die Virusextinktion identifiziert wurden, erklären zusammen 25% der phänotypischen Varianz. Dieser Genombereich wurde schon in anderen Pflanzen als für Resistenzmechanismen zuständig beschrieben. Die Ergebnisse der aktuellen Studie lassen deshalb vermuten, dass die BYDV-Resistenz oligogen vererbt wird und, dass Gene, die in generelle Resistenzmechanismen involviert sind, auch an der BYDV-Resistenz in Mais beteiligt sind.

Die GWAS hat den Vorteil, dass eine große Anzahl von Allelen pro Locus gleichzeitig untersucht werden können. Da in verbundenen Populationen eine höhere Rekombination stattgefunden hat, ist die Auflösung, verglichen zur klassischen quantitative trait locus (QTL) Kartierung, höher. Trotzdem können Gene, die zu einer großen phänotypischen Variation führen, unentdeckt bleiben, wenn sie eine niedrige Allel Frequenz aufweisen. Durch die ausgeglichene Allelfrequenz in biparentalen Populationen hat die klassische QTL-Analyse eine höhere Power weitere QTL zu detektieren als eine GWAS. Deshalb war ein weiteres Ziel dieser Arbeit (iv) die Validierung der Genomregionen in einer QTL-Analyse mit verbundenen Populationen.
Die Genomregion auf Chromosom 10, die in der GWAS identifiziert wurde, konnte in der QTL-Analyse mit verbundenen Populationen und in QTL- Analysen mit Einzel-Populationen validiert werden. Darüber hinaus kolokalisieren diese QTL mit den QTL, die in kontrollierten Gewächshausbedingungen identifiziert wurden. Außerdem lassen die Ergebnisse vermuten, dass die Gene auch an der Ausprägung anderer Virus-Resistenzen beteiligt sind. Da diese Genomregionen 45% der phänotypischen Varianz erklären, sind sie vielversprechend für die Marker-gestützte Selektion.

Die hohe genotypische Varianz, die in meiner Arbeit beobachtet wurde, stellte eine gute Gundlage für die Identifizierung von molekularen Markern

dar, die mit der BYDV Resistenz assoziiert sind. Diese Marker, welche mit einer GWAS identifiziert wurden, konnten mit einer QTL Kartierung verifiziert werden und sind vielversprechend für die markergestützte Selektion auf BYDV Resistenz in der Maiszüchtung.

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Erklärung

Hiermit erkläre ich an Eides statt, dass die vorliegende Arbeit von mir selbst verfasst und lediglich unter Zuhilfenahme der angegebenen Quellen und Hilfsmittel angefertigt wurde. Wörtlich oder inhaltlich übernommene Stellen wurden als solche gekennzeichnet.

Die vorliegende Arbeit wurde in gleicher oder ähnlicher Form noch keiner anderen Institution oder Prüfungsbehörde vorgelegt.

Insbesondere erkläre ich, dass ich nicht früher oder gleichzeitig einen Antrag auf Eröffnung eines Promotionsverfahrens unter Vorlage der hier eingereichten Dissertation gestellt habe.

Köln, im Januar 2015

Frederike Horn