Doctoral Thesis

Improvement of protein quality in waxy maize (Zea mays L.) by doubled haploid and marker assisted selection techniques

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Publication Date:
2010

Permanent Link:
https://doi.org/10.3929/ethz-a-006192803

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IMPROVEMENT OF PROTEIN QUALITY IN WAXY MAIZE (*Zea mays* L.) BY DOUBLED HAPLOID AND MARKER ASSISTED SELECTION TECHNIQUES

A dissertation submitted to
ETH ZURICH
for the degree of
Doctor of Sciences

presented by
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Zürich, 2010
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List of abbreviation

Ala  alanine
AM  amylose
ANOVA  analyses of the variance
AP  amylopectin
ASI  Anthesis – silking interval
C1  functional colored
c1  colorless
C1I  dominant inhibitor
CIMMYT  International Maize and Wheat Improvement Center
DHs  Doubled haploid
DMSO  Dimethylsulfoxide
DNA  Deoxyribonucleic acid
dNTP  deoxyribonucleotide triphosphate
EF1A  elongation factor 1α
Ex  Experiment
FAO  Food and Agriculture Organization of the United Nations
FCM  flow cytometry
f/2  floury
G1  gap1 phase (in interphase)
G2  gap2 phase (in interphase)
GBSSI  granule bound starch synthase I
I2/KI  Iodine/Potassium iodide
IL  Inbred line
Ileu  isoleucine
Leu  leucine
Lys  lysine
MAS  Marker assisted selection
Met  methionine
o2  opaque 2
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>OD</td>
<td>objective density</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>QPM</td>
<td>Quality protein maize</td>
</tr>
<tr>
<td>rpm</td>
<td>round per minute</td>
</tr>
<tr>
<td>SEA</td>
<td>South East Asia</td>
</tr>
<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
</tr>
<tr>
<td>su</td>
<td>sugary</td>
</tr>
<tr>
<td>Thre</td>
<td>threonine</td>
</tr>
<tr>
<td>Val</td>
<td>valine</td>
</tr>
<tr>
<td>wx</td>
<td>waxy</td>
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</tbody>
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Summary

Maize is one of the most important cereal crops for the industry as well as for human consumption since it is either directly consumed or used for meat production. According to statistics of the Food and Agricultural Organization of the United Nations (FAO), maize breeders were and still are very successful in improving maize grain yield; global maize yield worldwide increased from 1.9 tons/ha in 1960 to 5.1 tons/ha in 2008. Besides, maize breeders were also able to create new varieties with improved industrial or nutritional quality, e.g. with higher contents in amino acids or vitamins.

Known as the main source of carbohydrates for several populations, maize does not supply enough protein to cover human requirement. Thus, malnutrition is quite obvious for populations of many developing countries, where maize is consumed as a food staple. In Asia, a specialty maize called waxy maize, controlled by the waxy (wx) gene, is mainly used for human consumption while other flint, dent or flint*dent maize varieties are cultivated for feeding purposes. Waxy maize was first reported in China in 1909; it differs from other maize types in the starch composition of the endosperm, pollen and embryo-sac, which exclusively consists in amylopectin. Since the nutritional quality of waxy maize is similar to those of the other maize types, i.e. poor in protein quality and quantity, a diet based on waxy maize with no or limited other protein source is the main reason of malnutrition for many marginal populations like e.g. minority ethnic groups living in South East Asia.

The main nutritional deficiency of maize is the limited content in two essential amino acids, lysine and tryptophan. High quality protein maize, resulting from a mutation in the opaque2 (o2) gene, contained almost twice the amount of lysine and tryptophan of common maize. However, negative pleiotropic effects related to this mutation (low yield, soft and opaque kernels, susceptibility to pests and diseases) decreased drastically the interest of breeders in this type of maize. Fortunately, research done at CIMMYT permitted to counter these pleiotropic effects thanks to modifier gene(s) in so-called quality protein maize (QPM).

Improving the protein quality of waxy maize for ensuring better food resource in developing countries was the main goal of this project. Subtropical/tropical QPM maize were first crossed with local waxy maize in order to get waxy*QPM hybrids that were heterozygous
for both traits. Doubled haploid (DH) lines were derived from these plant materials and the double recessive \(wx-o2\) genotypes were selected. Implementing the technique of producing DH by \textit{in vivo} gynogenesis permitted to drastically reduce the time needed to get double recessive \(wx-o2\) inbred lines derived from heterozygous hybrids compared to conventional breeding methods.

An important part of the project consisted first in evaluating the feasibility of and optimizing current \textit{in vivo} gynogenesis procedure for subtropical and tropical plant material. Five different waxy\textsuperscript{*}QPM hybrids of various origins (Thailand: Kwpi\#4-1 x CLQ-RCYQ, Vietnam: Inco33Q3 and China: (CN\textsuperscript{13}, CN35 and CN37)) were pollinated with modern European inducer lines (RWS, RWK76 and RWK76RWS) in order to produce haploid lines. However, subtropical/tropical maize carried genetic factor(s) which complicated the visual selection of haploids according to anthocyanin pigmentation. Thus, selecting haploid seeds among the induced progeny took much longer than expected. Among the seeds selected visually, many false positives were eventually detected by flow cytometry. The eventual haploid induction rate obtained on subtropical/tropical plant material with modern inducer lines ranged from 9.6\% to 13.3\%, which was similar to those reported on European germplasms. Different methods for doubling the chromosome set of haploids were evaluated on diploid seedlings for technical reasons. The application of colchicine on young seedlings following Deimling’s method was the most efficient on this plant material with regard to both, chromosome doubling rate and plant fitness after treatment.

In a second part, the optimized process of haploid seeds selection and chromosome doubling was applied to produce DH lines from five Chinese heterozygous waxy\textsuperscript{*}QPM hybrids (CN\textsuperscript{13}, CN4, CN6, CN35 and CN37). Misclassifications according to visual selection of haploid seeds on the mean of anthocyanin pigmentation strongly confirmed the presence of anthocyanin inhibitor(s) in maternal subtropical waxy\textsuperscript{*}QPM donor hybrids. In general, haploid induction in hybrid CN3 was not only genetic but also environment dependent as it exhibited lower induction rates while CN35 and CN37 showed rates similar to the one found in first experiment.

The application of colchicine on haploid seedlings for chromosome doubling set was not comparable to results obtained with diploid seedlings in previous experiment. Even though

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\textsuperscript{1} Original names were B3, B4, B6, B35 and B37; however as B37 is the registered name, the names were modified to CN3, CN4, CN6, CN35 and CN37, with the agreement of the seed provider.
the survival and doubling rates were lower than previously described, the doubling rate controlled by flow cytometry ranged from 27.8% to 54.1%, which was acceptable when compared to the literature. Although the long anthesis silking interval of DH plants led to an eventual regeneration rate of less than 30% of the treated haploid plants, this study illustrated the possibility of applying this technique on subtropical/tropical waxy*QPM maize in order to produce rapidly inbred lines for breeding.

The main aim of this project was to improve the protein quality of waxy maize for human consumption. Therefore, developing an effective method for selecting for waxy and high protein quality traits in the five haploid populations generated (ETH3, ETH4, ETH6, ETH35 and ETH37 derived from CN3, CN4, CN6, CN35 and CN37, respectively) was an essential part of this work. The reliability of several markers for \( o^2 \) and \( wx \) was investigated. The two molecular markers phi112 and W4 distinguished genotypes of interest, i.e. homozygous for the recessive allele \( wx \) and \( o^2 \), respectively. As waxy maize starch consists in almost pure amylopectin, it can be discriminate from wild type maize at the reproductive stage by iodine staining of pollen. This permitted to control the results obtained with the newly developed marker W4 for \( wx \). Since pollen staining was deficient on three populations, ETH3, ETH4 and ETH6, only pollen staining results of DH populations ETH35 and ETH37 confronted to molecular marker data. In these two populations, the molecular marker W4 was reliable for selection of waxy genotypes and could be used in combination with phi112 to identify double recessive \( wx-o^2 \) genotypes among the DHs.

The evaluation of the quality of double recessive \( wx-o^2 \) genotypes with regard to amino acid and amylopectin contents was performed on five genotypes: two DH lines, ETH37.1 and ETH37.2, two \( wx-o^2 \) hybrids, ETH35.1*ETH37.1 and ETH37.3*ETH37.2, derived from DH lines, and the \( wx-o^2 \) backcross population ETH35.1ETH37.1*ETH37.1. The starch of these double recessive \( wx-o^2 \) genotypes consisted almost exclusively in amylopectin, which clearly indicated the expression of the waxy trait in this plant material. Furthermore, modifications in the amino acid profile, especially characterized by an increase in lysine content compared to their wild-type counterparts, illustrates the expression of the quality protein (\( o^2 \)) trait. Vitreous endosperm, one of the QPM-associated traits related to the expression of modifier genes, was not conserved in the five double recessive \( wx-o^2 \) genotypes evaluated in our study. This may highlight as well the importance of taking into account additional QPM traits when selecting
plant material of interest among DH populations generated for improving waxy maize quality by introgression of o2.

Nevertheless, the five double recessive \textit{wx-o2} genotypes are very promising plant material for further studies. Besides their content in lysine that was almost doubled, the quality of the whole amino acid profile was also improved and their specific kernel texture due to pure amylopectin starch is probably more acceptable, especially for Asian populations who used to eat waxy maize and were recalcitrant to pure QPM varieties in the past. Extending this project by generating more diverse double recessive \textit{wx-o2} plant material derived from various waxy and QPM source as well as further investigations on the agronomic and organoleptic quality of these double recessive genotypes will determine the contribution of such an approach to the improvement of food quality for many people in developing countries, especially pauper and ethnic minority groups.
Zusammenfassung


Das Hauptziel des Promotionsprojekts bestand in der Verbesserung der Proteinqualität von Wachsmais, um zu einer besseren Ernährungslage in Entwicklungsländern beizutragen.
Subtropischer und tropischer QPM Mais wurden zunächst mit lokalem Warchs Mais gekreuzt, um Wachs * QPM Hybriden zu erstellen. Diese waren heterozygot für die beiden Merkmale o2 und wx. Aus diesem Material wurden doppelt haploide (DH)-Linien gezogen, um daraus doppelt rezessive wx-o2 Genotypen zu selektieren. Im Vergleich zu konventionellen Zuchtmethoden beschleunigte der Einsatz der in vivo DH-Technik die Erstellung doppelt rezessiver wx-o2 Inzuchtlinien deutlich.


Die im ersten Teil des Projekts vollzogene Methodenoptimierung wurde im zweiten Teil des Projekts angewandt, um für weitere Studien DH-Linien aus fünf chinesischen Hybriden zu ziehen (CN3, CN4, CN6, CN35 und CN37). Allerdings enthielten die auf der mütterlichen Seite eingesetzten Wachs * QPM Hybriden Anthocyan-Inhibitoren, so dass die visuelle Bonitur zu häufiger Fehlklassifikation führte. Die Induktionsraten des Hybriden CN3 hing nicht nur genetisch sondern auch ökologisch ab, weil es niedriger Raten zeigte, während die Hybriden CN35 und CN37 gleich Induktionsraten mit denen des ersten Experiments haben. Analysiert mit Durchfluss-Cytometrie lag die Verdopplungsrate der haploiden Pflanzen mit 27,8 - 54,1 % in einem Bereich, der auch in anderen Studien angegeben wird. Die colchizinierten DH-Pflanzen wiesen häufig lange Intervalle zwischen männlicher und weiblicher Blüte auf, so dass die Regenerationsrate unter 30 % lag. Trotzdem illustriert diese Studie, dass es möglich ist, die in
**vivo** DH-Technik auf subtropischem und tropischen *wx*QPM Mais anzuwenden, um so rasch Inzuchtlinien für die Züchtung zu generieren.

Das Hauptziel des Projektes bestand darin, die Proteinqualität in Wachsmais für den menschlichen Verzehr zu verbessern. Deshalb wurde eine Methode entwickelt, die Wachsmais- und QPM-Eigenschaften in fünf haploiden Populationen zu selektieren (ETH3, ETH4, ETH6, ETH35 und ETH37; jeweils abgeleitet von CN3, CN4, CN6, CN35 und CN37). Mit Hilfe der molekularen Marker phi112 und W4 wurden Genotypen selektiert, die die rezessiven Allele *wx* und *o2* homozygot trugen. Im folgenden wurde die Stärkezusammensetzung der marker-gestützt selektierten *wx-o2* Pflanzen mit Hilfe einer Iod-Stärkefärbung überprüft: Nur die doppelt rezessiven Pflanzen der Populationen ETH35 und ETH37 wiesen die Wachsmais Eigenschaft auf. In den anderen Genotypen wurde das Merkmal nicht ausgeprägt.


General Introduction

1  World food situation

Combined with global climate change, higher food prices result in more than 40 million people on the world suffering from hunger and in an arising number of undernourished people that reached 963 million in 2008. The Food and Agriculture Organization of the United Nations (FAO) warned that this number may even increase in the future due to financial and economic crises. Around 907 million of undernourished people are living in developing countries, and nearly two-third of the starving persons are living in Asia, especially in countries like China, India, Indonesia or Bangladesh (FAO, 2008b). Children are the most affected by hunger and Asia is the only continent where almost half of the underweight children (46%) are younger than five years old (Fig1.a and b). One of the reasons of this high proportion is the poor diet as meat consumption is rather low there compared to the consumption of cereals.

2  China and South East Asia and their ethnic minority groups

South East Asia (SEA) is a sub-region of Asia grouping countries that are geographically located South of China, East of India and North of Australia. Currently, some countries of the SEA group, such as Singapore, Thailand, Malaysia and Vietnam, are rapidly developing, especially in large cities.

These countries are quite successful in reducing hunger and poverty for their populations. This success can be accounted to many changes in the daily diet, especially the reduction of the rice fraction and the increase in meat and milk consumption (FAO, 2008c).

According to geographical specificities, the population is not evenly distributed among China and the various SEA countries, and also among cities within a country. Most of the population concentrates in large cities and the majority of the populations living in marginal areas belong to minority ethnic groups. Beside global poverty and low educational level, the main problems of those minority ethnic groups are the access to land, water and markets (Huynh et al., 2002). For these populations, rice and maize still remain the staple foods by tradition and meat is only rarely consumed.
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In Vietnam for instance, minority ethnic groups represent only 14% of the population but 29% of the pauper. Among the 12 poorest provinces of Vietnam, where more than 60% of the population is below the poverty line, the minority ethnic groups represent more than half of the population (AUSAID, 2002). There, approximately 35 to 45% of the children under 5 years old and almost 40% of the children under 15 years old were considered as underweight in 2001 (Swinkels and Turk, 2006). When meditating on this alarming rate of malnutrition, the urgent necessity of improving the main protein uptake of these populations, especially of their infants and children, becomes obvious.

3 Specialty maize varieties - waxy maize and quality protein maize

3.1 Waxy maize

In Asia, with the exception of the new arising quality protein maize (QPM), the two different main types of cultivated maize are normal maize, so-called field maize, for animal feed and waxy maize for human consumption. Waxy maize, also called specialty “sticky” maize, was found in China in the early 1900s and was described as carrying an endosperm with a dull and waxy – like appearance. Waxy maize is not only a grain quality mutant, in many SEA countries like Vietnam, it is an important food crop or vegetable that is mainly grown for human consumption in up-land and, to a smaller extent, in central low-land areas. In the central low-land, hybrids and modern open-pollinated waxy varieties with a shorter life-cycle, an increased
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tolerance to pests and higher yields have been recently released meanwhile traditional waxy landraces still remain the major crops cultivated in up-land areas. Waxy maize is also used as a staple food and vegetable in other countries of SEA and in some regions in China.

The waxy endosperm trait was found to be controlled by a single recessive gene \( w_x \) located on the short arm of chromosome 9 (Coe et al., 1988). Thus, waxy maize is one specific mutant of normal maize; it differentiates from the wild type in the proportion of almost 100% amylopectin in its starch compared to around 70% in regular maize. The content in other components, like amino acids, remains similar. As early as 1956, it has been suggested that amylopectin contains three different types of chains. Even though the structure of amylopectin has been studied extensively, the exact arrangement of the chains within the amylopectin molecule is still not clear. Takeda et al (1988) reported a relatively high iodine affinity of the amylopectin (1.10g/100g) compared to those of starch containing both, amylopectin (AP) and amylose(AM). AM is known as a linear component with 1-4-\( \alpha \) glucosidic bonds, which can form coils around the iodine and turns the color of the iodine (potassium iodide, \( I_2KI \) solution

Fig 2.a. Absorbance spectra of starch - iodine complexes. Cuvettes contained: pure amylose (AM) (8 pg ml-1); pure amylopectin (AP) (32 pg ml-1); mix of amylose and amlopectin (AM + AP) (8 pg ml-l AM + 32 pg ml-l AP); iodine reagent only (20 pg ml-l). (Jarvis and Walker, 1993).

Fig 2.b. Absorption spectra of starch-iodine complexes in different plant species. Normal maize (a), normal rice (b), A. hypochondriacus No.76339-2 (c), waxy maize (d), glutinous rice (e) and A. hypochondriacus No. 76343-1 (f) (Sugimoto et al., 1981)
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to dark blue or black. The starch molecules of waxy genotypes (~100% amylopectin) are branched, with long external chains. Thus, after staining with iodine, the absorbance spectra of starch-iodine complexes differ in their peaks illustrating the AP and AM contents (respectively discriminating AM-free starch and starch containing AM) (Fig 2a) [Knutson, 1999; SenGupta, 1965]. Chromatographic profiles of starches of waxy plants revealed no amylose peak (Fig 2b).

Protein content of maize is rather low in both, quality and quantity. Maize proteins are divided into four different groups according to their solubility: albumins (purely water soluble), globulins (diluted salt solution soluble), prolamins (70% ethanol soluble) and glutelins (base or acid soluble) (Landry and Moureaux, 1980). The relative proportion of these components can vary according to the genetic background and/or to environmental conditions. Contributing to 80% to 85% of the maize kernel weight, the endosperm contains around 80% of the total protein content, meanwhile the embryo contributes approximately to 20% of the total protein content, 60% of which being albumins, with inter alia tryptophan and lysine amino acids (FAO, 1992; Vasal, S.K., 2001). From the human nutritional viewpoint, lysine is one of the most important amino acids, followed by tryptophan, not only for infants but also for adults. Proteins of maize endosperm consist in around 3% of albumins, 3% of globulins, 34% of glutelins and between 44 to 79% of zeins. Zein is a specific class of prolamin proteins. The zein class contains four distinct types of proteins referring to their solubility and their size: α-zeins (22 and 19 kDa), β-zeins (14 kDa), γ-zeins (27 and 16 kDa), δ-zeins (10 kDa) (Esen, 1986; Viotti et al., 1985; Wallace et al., 1990). The genes encoding the storage of maize proteins are present as multiple copies, whose estimated number ranged up to 100 genes (Burr and Burr, 1981). The genes are distributed on several chromosomes over the maize genome. Among the zein fractions, α-zeins, encoded by a large gene family, are the most abundant fraction and represents up to 80% of the total zeins. The other fractions like β-, γ-, δ- zeins are encoded by only one or two genes.

The lysine content of zeins is low, only 0.1g/100g of proteins; the tryptophan content is also quite low in zeins. Deficiencies in lysine and tryptophan related to a diet based on maize have been demonstrated in other studies on animal and human nutrition (Bressani et al., 1969; FAO, 1992). The deficiency in lysine and tryptophan is the main factor of malnutrition for people who consume maize as staple food in general and waxy maize in China and SEA particularly. The question of improving the protein intake of these populations through the improvement of their staple food with regard to protein quality arises.
3.2 Quality protein maize (QPM)

Since maize is a cereal of poor nutritional quality but one of the most important food sources for developing countries, breeding toward maize with enhanced protein quality began in the mid of the 1960s, when mutants with an increased lysine content, like opaque2 (o2) and floury2 (fl2), were discovered. The opaque2 gene was first mentioned by Singleton and Jones in the 1920s as reported by Emerson et al. (1935), but the nutritional significance of this mutant was first pointed out 40 years later by Mertz et al. (1964) and led to the development of high quality protein maize (QPM). The opaque2 mutation nearly doubles the lysine content of the maize endosperm. This increase in lysine observed in o2 mutants results from several phenomena (Moro et al., 1996): (i) the reduction in the synthesis of zein storage proteins, which do not contain lysine, (ii) the enhancement of the synthesis of numerous other non-zein proteins, the most of which contain lysine, a general increase in the level of free amino-acids, including lysine. Moro et al (1996) reported that the elongation factor 1α (EF1A) was increased in o2 mutants, and that its concentration was highly correlated with the lysine content of the endosperm \( r = 0.9 \). Even though the EF1A itself is rich in lysine (10%) (Sun et al., 1997), in general it accounts only for 2% of the lysine of the maize endosperm. Lopez–Valenzuela et al. (2004) reported that the levels of cytoskeletal proteins such as actin or tubulin were increased in o2 inbred lines with high EF1A content.

The increase of two amino-acids, lysine and tryptophan, in o2 maize results from an alteration of the relative amounts of different fractions constituting the maize endosperm proteins. The zein or prolamin fractions are reduced substantially, thus increasing the relative part of other fractions. Since zein is practically devoid of lysine, while other fractions are richer in lysine, the overall amino acid profile exhibits an increase in lysine. No new proteins are formed and the composition of the different protein fractions remains unaffected (Vasal, S.K., 2001). The ribonuclease activity is several times higher in o2 mutants, compared to the wild type (Dalby and Cagampang, 1970; Wilson et al., 2004). This activity cannot be regarded as the principal factor for reducing the zein accumulation in the endosperm but as a secondary effect coupled to the o2 mutation. Furthermore, the endosperm of this mutation changes in several soluble proteins (Larkins et al., 1982). The o2 mutation also affects several agronomic traits including kernel characteristics and grain yield. The lower yield caused by an increased endosperm size that is soft chalky and dull usually results in more easily damageable kernels,


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and thus in a higher susceptibility to pests and/or fungal diseases (Vasal, S.K., 2001). Various methods have been explored in order to overcome the ‘defects’ of o2 maize.

Double mutant combinations involving either different high lysine mutants or a high lysine mutant combined to some other endosperm mutant(s) were attempted to alter at most unacceptable properties of standard soft opaque2 kernels. Two combinations (opaque2-floury2 (o2o2fl2fl2) (Nelson, O.E., 1966) and sugary2-opaque2 (su2su2o2o2)(Garwood and Creech, 1972; Glover et al., 1975), still showed a reduction in grain yield and their kernels phenotype was still not satisfying (Roundy and Glover, 1975). It would require additional selection toward more uniformity; although the kernels were translucent, their apperance was somewhat dull.

The integration of the o2 gene into a serie of maize inbred lines (IL) by repetitive backcrosses resulted in a wide range of different endosperm textures of o2 genotypes. While some IL produced completely soft kernels, others had particularly hard kernels; these differences appeared to be related to the modifier gene(s) for o2 (Vasal, S.K, 1975; Vasal, S.K., 1999). The o2 germplasm with hard endosperm was named high quality protein maize (QPM) by the International Maize and Wheat Improvement Center (CIMMYT, Mexico), in opposition to the soft endosperm o2, called standard o2.

In general, QPM varieties of maize contain about 55% more tryptophan, 30% more lysine and 38% less leucine than the wild type. Another important factor is the good biological value of QPM, which refers to the amount of absorbed nitrogen needed to provide the amino acids necessary for different metabolic functions. Similar result could not be obtained with normal maize even by using twice the amount of nitrogen (Vasal, S.K., 2001). The nitrogen balance index is the difference between the dietary intake of nitrogen (mainly proteins) and its excretion (as urea and other waste products). In o2 maize proteins, this index is close to those of skim milk (0.72 and 0.8, respectively); it was demonstrated that o2 maize has 90% of the nutritive value of milk protein for young children (FAO, 1992).

4 **Maize haploids and their application in breeding**

4.1 **Haploid productions**

The production of haploids means producing sporophytes with a gametic number of chromosomes (1n). The term haploid refers to the number of chromosomes set carried by the
Introduction

plant, which is commonly used to distinguish haploid from regular sporophytes (Blakeslee and Belling, 1924). Haploids can arise through various processes like parthenogenesis, pollen irradiation, selection of seed with twin embryos, sparse pollination, alien cytoplasm, wide hybridization or through the use of certain specific genetic stocks (in maize for instance) (Maluszynski et al., 2003).

The occurrence of spontaneous haploid plants has been reported in many angiosperm species (Gordon and Riley, 1963; Lashermes and Berkert, 1988), especially in species of agricultural importance like *Triticum* or *Nicotiana*. In maize, natural haploids were discovered in the early 1900s (reported in 1929 by Stadler and Randolph as mentioned later by Randolph (1932)). Until now, the mechanisms of haploids induction are still unclear. Doubled haploid (DH) are diploid plants resulting from haploid plants, the chromosomes set of which has been doubled. Chromosome doubling permits to regenerate and thus to maintain haploid plants which otherwise would usually be sterile. The application of doubled haploids in maize breeding was

Fig 3. Anatomy and life cycle of maize. The life cycle of a maize plant, including the diploid sporophytic stage and the haploid gametophytic stage. Source: (Walbot and Evans, 2003) (reprinted with the permission of the authors and of Nature Publishing Group)
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limited until Chase developed pure elite doubled haploid lines, which were used in the production of commercial hybrids (Chase, 1947, 1949b). Chase was the first breeder, who succeeded in selfing haploid maize and showed that the step of chromosome doubling might even not be necessary if the rate of natural chromosome doubling was high enough (Chase, 1949a, 1963).

In maize, haploids can be produced in several ways: spontaneous induction; genetic induction, environmental impact, artificial induction. Artificial induction is the most practical method for breeding programs; it bases on anther, embryo and microspore culture or on breeding/crossing different species and on chromosome elimination or apomixis. So far, two different types of haploids depending on embryo’s derivation are known: maternal haploids and paternal haploids, where the sporophyte develops from an unfertilized egg cell (gynogenesis) or sperm cell (androgenesis), respectively. In vitro androgenesis methods, where haploid plants derive from anther cultures, have been developed several decades ago. On the other hand, some natural lines, called inducer lines, which were able to produce maternal haploids when used as the pollinator were identified; this last haploid induction process is called in vivo gynogenesis. However, these inducer lines do not exclusively generate kernels with a haploid embryo after fertilization, the majority of the progeny is diploid (hybrid between the two parental lines). The haploid induction rate of modern inducer lines ranges from 5 to 8 % (Geiger, 2009). In vivo gynogenesis is now applied by almost all maize leading breeding companies because it was found to be the most efficient way to achieve a homozygous progeny (after the step of chromosome doubling) in shorter time than by traditional inbreeding methods.

4.2 Inducer lines

In maize, the normal fertilization occurs when one of the sperm nuclei fertilizes the egg cell to form the embryo and the other fuses with the diploid central cell (the two polar nuclei fusion) to form the triploid endosperm (Fig 3). A maize inducer line is a specific line that can break the double fertilization and eventually produce haploid seeds. The first maize haploid inducer line was described in 1929 and exhibited a very low rate of spontaneous haploid induction of 0.1% (Chase, 1949a). In the last decades, inducer lines have been intensively studied for maize breeding and several lines with enhanced induction rates were developed. In the 1960s, Coe developed new inducer lines originating from maize stock 6 with a haploid induction rate of up to 2.3% (Coe, 1959). Another inducer line, WS14, developed by Lashermes
and Beckert (1988) derived from a cross between the lines W23ig and Stock6, showed haploid induction rates of 3 to 5%. Chalyk [, 1999 #100] and Sarkar et al. (1994; 1972) found higher haploid induction rates when using a cross between Stock6 and Russian/Moldavian germplasm. The inducer line RWS (Röber et al., 2005), a modern European inducer line developed at the University of Hohenheim (Germany) was derived from F5 plants of a cross between the Russian induction synthetic KEMS and the French inducer line WS14; it exhibits a haploid induction rate of up to 8.1%.

![Figure 4. Haploid induction by in vivo gynogenesis and illustration of the method for selecting haploid seeds among the progeny according to the anthocyanin pigmentation of the embryo](image)

How inducer lines do produce seeds with haploid embryos still remain unclear. Wedzony et al. (2002) investigated the mechanism of chromosome elimination in the modern European inducer line, RWS, and pointed out that the micronuclei in the cytoplasm of every cell of the shoot primordium were of variable size, and that these micronuclei were characteristic for wasted chromatin having been eliminated from the cell in subsequent cell divisions. Gernand et al [, 2004 #90] observed, in embryonic cells, the degeneration of the chromosomes of the inducer line, which were fragmented a few days after fertilization.

In this project, three different inducer lines, kindly provided by Prof. Geiger H.H. from the University of Hohenheim (Germany), were used: RWS, RWK76, and RWK76RWS. These three inducer lines carry the gene R1-nj for anthocyanin pigmentation, which colors both compartments of seeds of the inducer lines, the endosperm and the embryo, red (Fig 4). In the progeny, all the seeds that carry a red triploid endosperm (derived from both parental genetics) but differ in the color of the embryo: embryos of diploid seeds are red (presence of the genetic
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of the inducer line), embryos of haploid seeds are white (presence only of the genetic of the maternal donor plant).

4.3 Chromosome doubling

Haploid plants are usually less vigorous and more sensitive to diseases or biotic and abiotic stress. They carry only one set of chromosome and therefore are sterile. According to Geiger (2006) (and also personally observed) haploid products derived from the inducer line RWS are mostly male-sterile though mostly female-fertile. In order to maintain the genes of interest present in the haploid plant and to use the genetic background for breeding purposes, the chromosome set has to be doubled.

Spontaneous cases of chromosome doubling were observed for haploid plants in which fertile diploid sections occurred spontaneously at a low rate (Chang and Coe, 2009). Thus, the tassel of haploid plants did produce some fertile and non-fertile anthers, which were often not able to dehisce and/or release pollen grains. Depending on the genotype, between 2.8 and 46.0% of haploid plants shed pollen grains but only half of that showed a larger tassel sector with normal anthers (Chang and Coe, 2009; Geiger, 2009). On the female flower side, the rate of spontaneous occurrence of fertility is much higher, ranging from 25.0 to 94.0%.

Besides spontaneous doubling, haploid plant chromosome set can be doubled by antimitotic chemicals (like colchicine or oryzalin) or by nitrous oxide gas. Four different mechanisms for doubling plants’ chromosome set were described (Segui'-Simarro and Nuez, 2008): endo-reduplication (DNA duplication without mitosis), nuclear fusion (merging of coalescing nuclei into a larger nucleus, mixing both DNA contents), endo-mitosis (mitosis in the absence of both, mitotic spindle and nuclear envelope breakdown) and C-mitosis (colchicine – induced collapse of the mitotic spindle and nuclear envelope breakdown).

Among the different pathways for doubling the chromosome set in the process of generating maize doubled haploids, the application of colchicine was identified as the most effective method. With high colchicine concentration, the mitosis is blocked at the metaphase (C-metaphase) of the cell division cycle, which eventually leads to a G1 cell with a doubled number of chromosomes compared to the G1 cell of origin (Segui'-Simarro and Nuez, 2008). Cells in C-metaphase were widely used for cytogenetic analysis. At lower concentration, sister chromatids could detach from each other. Three main factors, concentration, time and genotype,
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influence the most the success of chromosome doubling experiments with colchicine (Castillo et al., 2009).

The production of DH was initiated quite early for maize breeding, since the first maize haploid plants had been discovered in 1929. Until now, the process of producing DH developed as a very important technique implemented by most of the international breeding companies. Several methods of chromosome doubling have been published and were very helpful for breeders:

- Deimling’s method (Deimling et al., 1997): a small part of the coleoptile tips of haploid seedlings (with 1cm long colepotile) is removed and the whole seedlings are placed into a 0.06% colchicine and 0.5% dimethylsulfoxide (DMSO) solution for 12h. These seedlings are planted on the field after having been washed under tap water for 20min. This method was applied on field scale and showed statistics of 72.5% of survival plants, 50% of fertile plants, 39% of plants which could be self-pollinated and 27.3% of plants eventually producing seeds.

- Zabirova’s method (Zabirova et al., 1996): a solution of 0.125% colchicine and 0.5% DMSO is directly injected into young haploid plants (3rd – 4th leaf stage) at a point of 3 to 5mm above the apex. This method was applied both, in greenhouse and on the field, where 88.6% (on field) and 92.8% (in greenhouse) of the plants survived, 42.4% shed pollen, 30.5% could be self-pollinated and 11.9% of the plants delayed shedding of pollen and silk emergence. In the field, only 16.1% plants were male-fertile but this low rate was described as being a consequence of unusual low temperatures.

- Kato’s method (Kato, 2002): haploid plants at the 6th leaf stage are treated by nitrogen gas (N\textsubscript{2}O) for 2 days. By this method, 44% of the plants were able to produce seeds. Although this method seems very efficient and attractive over the previous one, it requires special conditions we could to apply with our facilities.

- Bordes’ method (Bordes et al., 1997): young plants at the 3rd leaf stage were grouped in homogenous bunches of 20 seedlings, which were put into a colchicine solution (1.5g/l) for 3h. At the end, 30-61% of the plants were able to produced seeds.

4.4 Advantages and disadvantages of doubled haploid techniques

Doubled haploids (DH) are now widely implemented in maize breeding by many international breeding companies because it combines several advantages (Chang and Coe, 2009; Geiger, 2009): (i) it provides homozygosity in only one generation (i.e. about 7 months)
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compared to 6 to 8 generations (i.e. 3 to 4 years) by conventional breeding; (ii) it can enrich the genetic pool in favorable genes or eliminate harmful and unfavorable genes; (iii) it also provides a broad source of material for genetic mutations; (iv) DH lines are routinely used to produce mapping populations for mapping simple genetic traits of agronomic importance like resistance to disease and plant stature.

However, the application of the method of producing DH is still limited by the low haploid induction rates provided by current inducer lines and marker systems that are not always precise and efficient to detect the haploids among the putative ones as well as low chromosome doubling frequencies (around 10%). The major problems limiting the use of the DH technique are still remaining technical difficulties, and high cost of this method.

**5 Goals of the PhD project**

The main goal of this project was to obtain rapidly homozygous lines of improved waxy maize with regard to the protein quality by applying the DH technique to hybrids between waxy populations and QPM lines and by the selection of double recessive $wx-o2$ DH genotypes. The different methods available in the literature to achieve this were evaluated and eventually improved by investigating the best conditions for haploid induction and chromosome doubling of subtropical/tropical waxy maize, which was another goal of this doctoral work. At the end, the nutritional quality with regard to the amino acid profile of the double recessive DH or populations derived from the DH was evaluated as well as the amylpectin content in heterozygous and homozygous seeds derived from waxy*QPM combinations. This last point may bring also some light into the impact of the unknown modifier genes for opaque2 of QPM developed by CIMMYT to counter negative pleiotropic effects of the $o2$ mutation.
Chapter 1

Induction of haploid seeds derived from tropical and subtropical waxy*QPM hybrids by modern European inducer lines

* A publication based on this chapter and chapter 2 has been submitted

Abstract

The implementation of modern inducer lines in maize breeding can substantially decrease the time needed for creating elite inbred lines. In industrialized countries, this method almost replaced conventional backcross methods. However, the application of in vivo gynogenesis for inducing doubled haploids is still limited to European and US maize germplasms and as to be adapted/improved for exotic plant material. In this chapter, the reliability of three modern European inducer lines (kindly provided by Prof. Geiger H.H., University of Hohenheim), RWS, RWK76 and RWK76RWS, to produce haploid progenies of subtropical/tropical specialty maize was investigated. The maternal donor plants were hybrids between waxy maize and quality protein maize originating from Thailand (Kwpi#4-1 x CLQ-RCYQ), Vietnam (Inco33Q3) and China (CN23, CN35 and CN37). The classical visual haploid selection method was not reliable with this subtropical/tropical plant material as the maternal donor since many false positives were assessed among the induced seeds selected according to anthocyanin pigmentation when controlling the ploidy level by flow cytometry; almost 50% of the induced seeds showed an inhibition of anthocyanin expression. Eventually, the haploid induction rates ranged from 9.6% to 13.3% of the induced seeds. The step of chromosome doubling that follows usually the haploid induction to generate doubled haploids was studied as well and the efficiency of different methods of chromosome doubling was evaluated on subtropical/tropical maize material. Deimling’s method of chromosome doubling, where the seedlings are immersed for 12h in a solution of 0.06% colchicine and 0.5% dimethylsulfoxide (DMSO), proved to be the most suitable for plant material of this origin as well.

1.1 Introduction

1.1.1 Maize haploids and in vivo haploid induction

2 Original names were B3, B35 and B37; however as B37 is the registered name, the names were modified to CN3, CN35 and CN37, with the agreement of the seeds provider.
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In modern maize breeding, different techniques for producing doubled haploid (DH) lines are commonly applied as main methods for rapidly creating new varieties. In some major seed companies, DH lines are about to replace inbred lines produced by conventional backcross selection. Haploid plants can be produced by both, *in vitro* and *in vivo* methods. However, maize is a recalcitrant species for *in vitro* culture (callus formation and embryogenesis) and its maintenance in tissue culture is very genotype dependent; only few genotypes were found to be appropriate for *in vitro* embryogenesis (Geiger, 2009). *In vivo*, maize haploids are produced mostly by crossing the genotypes of interest with specific inducer genotypes, the so-called inducer lines. *In vivo* haploid induction can be either paternal or maternal; paternal induction occurs when the inducer line acts as the pollen receiver and maternal when the inducer line acts as the pollen donor. Compared to *in vitro* techniques, *in vivo* haploid induction procedures appear to be less complicated and more reliable, which does not mean that they are not still improvable. *In vivo* techniques of haploid induction have been widely implemented over the last 10 to 15 years for generating DH lines (Geiger, 2009).

History of maize natural haploidy started in the early 1900s as it was first reported in 1929 by Stadler and Randolph and later cited by Randolph (1932). Nevertheless, the implementation of doubled haploids in maize breeding was limited until the beginning of the 1950s, when Chase first developed pure elite DH lines for the production of commercial hybrids (Chase, 1949a). Until now, the mechanisms leading to the development of haploid embryos in maize are still unclear. In maternal haploid induction, one of the two sperm cells seems not to fuse with the egg cell, while the other fuses with the two polar cells leading eventually to a haploid embryo and triploid endosperm. Paternal haploid induction rates are around 1 to 2% (Belicuas et al., 2007) while maternal haploid induction rates are higher (around 8% with modern European inducer lines) (Geiger, 2009). *In vivo* haploid induction has been improved drastically in the last 30 years by the discovery/development of more efficient inducer lines. Most of the efforts were devoted to the improvement of the maternal system, the haploid induction rate of which increased from around 2.3% in the 1960s (Coe, 1959) to 3 to 5% in the late 1990s (Lashermes and Berkert, 1988), 6% in 2000s (Sarkar, K.R. et al., 1994) to even up to more than 8% with the modern European line RWS (Röber et al., 2005)

1.1.2 Selection of haploids seeds

1.1.2.1 Anthocyanin pigmentation
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The key issue for applying in vivo haploid induction is the selection of haploid seeds among the progeny. To facilitate the selection of haploid seeds, modern inducer lines carry the $R1{-}nj$ gene, which causes a “red crown” endosperm as well as a red (or purple) embryo. After maternal donors were pollinated by inducer lines, haploid seeds combine a haploid maternal embryo (colorless) and a triploid endosperm (red crown aleurone) derived from both, the maternal donor and the inducer line (Geiger, 2009). In diploid seeds, the embryo is red as well since half of its genome derives from the inducer line. Nanda and Chase (1966) were the first breeders who used this gene as a visual marker for selecting haploid seeds in the process of generating DH lines. The $R1{-}nj$ gene became an essential factor in haploid seeds identification. However, the anthocyanin pigmentation is influenced by the environment and the genetic background of the donor plant, which renders it a pretty complex and sometimes unreliable system (Cone, 2007). Besides, the use of the transgenic herbicide (BASTA®) resistance marker was tested by Geiger et al (1994) and found to be reliable for the selection of haploid seeds but was not further used since its utilization was too labor intensive.

1.1.2.2 Flow cytometry

Flow cytometry (FCM) is a technology, which simultaneously measures and analyses multiple physical characteristics of single particles, usually cells, as they flow in a fluid stream through a beam of light. This technique was developed for biological purposes following photometry, spectrometry and fluorometry techniques applied by physicists and chemists (Howard, 2007). FCM analyses optical properties such as fluorescence and light scatter of microscopic particles in liquid suspension. Cells and cell organelles are individually measured at high speed. With this technique, single cells can be evaluated in high throughput, classified as required and sorted into single cell units or homogenous populations. The application of FCM in plant science started in the 1970s, where it was mainly applied for ploidy analysis and cell cycle studies as it is convenient and rapid tool for screening fresh and dried plant samples (Suda et al., 2007). FCM is used in plant science to estimate the DNA quantity (C-value) in absolute units (DNA picograms and number of base pairs) as well (Dolezel et al., 2007). The relationship between ploidy and nuclear DNA content makes the assay suitable for the determination of the ploidy level and the detection of mixo- or aneuploidy.

1.1.3 Chromosome doubling after in vivo haploid induction

$^3$Registered trademark, Bayer crop science
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Since haploid plants are sterile, doubling the chromosome set becomes the most important step after having selected haploid seedlings. In nature, chromosome doubling can happen spontaneously. The frequency of spontaneous fertility of the female inflorescence in haploid maize is quite high, ranging from 25 to 96% while spontaneous male fertility is much rarer (Chang and Coe, 2009; Geiger, 2009). In maize, artificial chromosome doubling by colchicine treatment became popular since it enabled to successfully self-pollinate plants, which were originally haploids (Chase, 1952). Several methods for doubling the chromosome set with colchicine were tested. In general, the doubling rate observed in tassels of haploid plants treated with a solution of 0.06 to 0.50% colchicine reached 20 to 50% (Han et al., 2006). Seaney (1955) treated haploid plants by immersing the whole root system in the colchicine solution. With a solution of 0.05% colchicine, the self-fertility was significantly improved; higher colchicine concentrations were rather deleterious for seedlings and caused severe injury and very low recovery rate. Deimling et al. (1997) and Zabirova et al. (1996) applied colchicine at the seedling stage with higher colchicine concentrations of 0.060 to 0.125% and obtained a significant number of doubled haploid plants. Kato (2002) used nitrous oxide gas (N₂O) on maize at the 6th leaf stage, increasing the doubling rate to up to 44%, but this method is very expensive and requires specific facilities. Until now, colchicine is still reported as the most efficient chemical to double the chromosome set in maize. However, the efficiency of the colchicine and the ideal concentration are genotype dependent; no common method could give the best result for all genotypes (Castillo et al., 2009).

1.1.4 Waxy maize and quality protein maize (QPM)

In South East Asia (SEA), many minority ethnic groups are mainly located in up-land or marginal areas. The specialty waxy maize is consumed as the staple food in up-land regions but in low-land areas it is cultivated as an additional vegetable. Waxy maize, characterized by a starch consisting in almost pure amylopectin, is controlled by a single recessive \( wx \) allele of the \textit{waxy} gene, located on the short arm of chromosome 9 (Coe et al., 1988). Waxy starch composition regarding non carbohydric components, like amino acids, remains similar to those of regular maize: low in quantity and poor in quality; both are particularly deficient in lysine and tryptophan, which are, in the viewpoint of human nutrition, the most important amino acids, not only for infants but also for adults (WHO, 1985). Thus, the consumption of maize, especially of waxy maize as a staple food in SEA, often leads to malnutrition issues.
In the middle of the 1960’s, maize genotypes with higher protein quality, and especially higher lysine content, caused by mutations of the \textit{opaque2} (\textit{o2}) and/or \textit{floury2} (\textit{fl2}) genes were discovered (Mertz et al., 1964). However, these mutations also affect several agronomic traits including kernel characteristics that negatively impact grain yield; these genotypes exhibited soft, chalky dull and large endosperms, which render the kernels easily damageable and highly susceptible to pests and/or fungal diseases (Vasal, S.K., 2001). These negative traits could be overcome by specific modifier gene(s) for \textit{o2} that lead to the production of \textit{o2} kernels of particularly hard and resistant phenotype. Maize that carries both, the \textit{o2} and the \textit{modifier} alleles was developed by CIMMYT and named high quality protein maize (QPM) (Prasana et al., 2001). In the last decades, QPM became an important part of maize breeding programs in many developing countries.

Basically, the most common method for breeding a new waxy maize variety in SEA is the method of conventional cross/backcross/self-pollination. With this method, it is admitted that transferring one specific gene into another specific genetic background (98\% of homozygous genes) requires around six generations (Tracy, 2004). Doubled haploid production is a promising approach to obtain rapidly homozygous plants for maize breeding programs but its application to tropical and subtropical waxy maize is still very limited. The efficiency of haploid induction seems very variable, depending on the inducer line but also on the maternal donor material, which plays a determining role in the success of the haploid induction.

In this chapter, the objectives were, first to find the best method for identifying haploid seeds derived from tropical and subtropical waxy*QPM hybrids after haploid induction with different modern European inducer lines and, second, to determine the optimal method for doubling the chromosome set of this material.

\section*{1.2 Plant materials}

\subsection*{1.2.1 Inducer lines}

Three different inducer lines, RWS, RWK76, and RWK76RWS (kindly provided by Prof. Geiger H.H. from University of Hohenheim, Germany), were used to generate haploid plant material. These lines were derived from F5-generations of the Russian inducer KEMS (Shatskaya et al., 1994) and of the French line WS14 (Lashermes and Berkert, 1988). Like most of the inducer lines, these three lines carry the \textit{R1-nj} marker gene for red crown endosperm and
red embryo. In addition, they carry the sun-independent gene \( PII \), the expression of which leads to a red colored stem at the late seedling stage. This should allow breeders to select easily the haploid plants out of false positive cases.

1.2.2 Maternal donor plants

Five waxy*QPM hybrids derived from crosses between waxy and QPM lines (i.e. heterozygous for both genes) were used as donor parents: Inco33Q3 (later called VN33, kindly provided by Dr. Le H.H., Agricultural Genetics Institute, Vietnam), CN3, CN35, CN37 (kindly provided by Dr. Wen R., Guangxi Maize Research Institute, China) and Kwpi#4-1 x CLQ-RCYQ (later called TH6, kindly provided by Dr. Jampatong S., Thailand National Corn and Sorghum Research Center, Thailand). These hybrids were hand-pollinated with pollen of the inducer lines to generate haploid seeds. The resulting seeds were used for evaluating and optimizing the haploid seeds selection method.

Since the stock of haploid seeds was limited, the evaluation of different methods of chromosome doubling by colchicine treatment (Table 1.1) was performed on diploid plant material of five heterozygous Chinese waxy*QPM hybrids, CN3, CN4, CN6, CN35, CN37.

1.3 Methods

1.3.1 Selection of haploid seeds

1.3.1.1 Visual selection according to anthocyanin pigmentation

Theoretically, the identification of haploid seeds after induction is facilitated by the “red crown” pigmented endosperm (triploid endosperm, where the paternal genome of the inducer line is represented) and unpigmented embryo (haploid embryo, where only the maternal genome is represented).
In order to clearly identify haploid seeds among the progeny, the seeds obtained after induction were divided into 3 bulks: (i) bulk 1 of unpigmented seeds (unpigmented endosperm and unpigmented embryo), (ii) bulk 2 of putative haploid seeds (red endosperm, unpigmented embryo) and (iii) F1 diploid seeds (red endosperm and red embryo) (Fig 1.1). All seeds with pigmented embryos were considered as diploid seeds (F1 hybrid between the maternal hybrid and the inducer line). Only seeds with unpigmented embryo, bulk 1 and bulk 2, were further considered. Since the situation of bulk 1, unpigmented seed, should not occur in theory, we hypothesized that the maternal genome may interfere in the expression of the anthocyanin pigmentation in both, the endosperm and the embryo, which complicates the visual selection of haploids.

The three modern inducer lines carry the sun-independent Pl1 gene, resulting usually in a diploid induced progeny with a red stem whereas the stem of haploid seedlings remains green. This should help to easier identify haploid plants, especially for genotypes, where the classical anthocyanin selection was not reliable. Unfortunately, the waxy maize materials and thus waxy*QPM hybrids as well in this project expressed the sun-independent red stem trait, which made this marker an unreliable marker for selecting haploid plants among the induced progeny, too. Since the visual selection of haploids among unpigmented seeds was impossible, the ploidy level of these seeds was determined by flow cytometry.

Fig 1.1. Maternal haploid induction scheme with possible progeny seed population
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1.3.1.2 Flow cytometry

Flow cytometry (FCM) allows identifying effectively haploid seedlings among the induced progeny. Since the selection of haploid seedlings is only the first step of the process of developing DH, it has to be non-destructive. The ploidy of putative haploid seeds (bulk 2) as well as of unpigmented seeds (bulk 1) was evaluated after germination (three to four days at 28°C). A small tip of the first leaf (just out of the coleoptile) was cut, chopped into small pieces with a razor blade and stained with 2ml DAPI (4',6-diamidino-2-phenylindone) solution (5µg/ml, Partec GmbH, Germany). After filtration through a nylon membrane (50µm mesh size), the filtrate was analyzed by FCM, at a par gain FL1 (fluorescence) of 420 to 430 nm (relative fluorescence - RF). A peak set at 60 and 130 FL (corresponding to nuclei in growth phase 1 (G1) and growth phase 2 (G2), respectively) illustrates diploid material while a peak set at 30 and 70 FL corresponds to haploid plant material (Fig 1.2).

![Flow cytometry spectra of haploid and diploid seedlings](image)

Fig 1.2. Flow cytometry spectra of haploid and diploid seedlings. G1 and G2 peaks illustrate the number of nuclei in growth phase 1 and growth phase 2, respectively

1.3.2 Chromosome doubling

In maize, colchicine was reported to be the most efficient chemical to double the chromosome set in vitro (Saisingtong et al., 1996) as well as in vivo. According to Deimling et
al. (1997) and Eder and Chalyk (2002) for the latter system, optimal colchicine concentrations for chromosome doubling vary from 0.060% to 0.125%. Different treatments based on the methods of Deimling et al. (1997) and Zabirova et al. (1996) were evaluated (Table 1.1). This experiment was duplicated with 10 seedlings per genotype for each treatment.

In experiment (Ex.) 1, the whole coleoptile was immersed in a solution of 0.06% colchicine and 0.50% DMSO (Dimethylsulfoxide) for 12h. Ex.2 and Ex.3 were alternative treatments, where only the root system, respectively the shoot, was immersed in the same solution as in Ex.1 for 12h. A control treatment (Ex.5), where the whole seedling was immersed in water for the same duration was conducted as well. In Ex.4, the colchicine solution (0.125%) was directly injected into the plant, following the method of Zabirova et al (1996). All the plants in Ex.1, Ex.2, Ex.3 and Ex.5 were washed under running tap water for at least 20min after the treatment. Since haploids were usually weaker than diploid plants and even more after colchicine treatment, they became more sensitive to fungi and diseases.

Table 1.1. Description of different colchicine treatments with the stage and part of the seedling treated, the concentration in colchicine and dimethylsulfoxide of the solution, and the duration of immersion. Washing indicates whether the seedlings were washed under tap water after treatment or not.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Treatment stage</th>
<th>Treatment part</th>
<th>Colchicine concentration (%)</th>
<th>DMSO (%)</th>
<th>Duration (hours)</th>
<th>Washing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ex.1</td>
<td>Coleoptile</td>
<td>Whole coleoptile</td>
<td>0.060</td>
<td>0.5</td>
<td>12</td>
<td>Yes</td>
</tr>
<tr>
<td>Ex.2</td>
<td>Coleoptile</td>
<td>Roots</td>
<td>0.060</td>
<td>0.5</td>
<td>12</td>
<td>Yes</td>
</tr>
<tr>
<td>Ex.3</td>
<td>1st leaf emergence</td>
<td>Shoot</td>
<td>0.060</td>
<td>0.5</td>
<td>12</td>
<td>Yes</td>
</tr>
<tr>
<td>Ex.4</td>
<td>3-4 leaves</td>
<td>Meristem</td>
<td>0.125</td>
<td>0.5</td>
<td>Punctual injection</td>
<td>No</td>
</tr>
<tr>
<td>Ex.5 (control)</td>
<td>Coleoptile</td>
<td>Whole coleoptile</td>
<td>0.000</td>
<td>0.0</td>
<td>12</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Thus, the soil was sterilized with 0.5% Algicid (Alkyl – dimethyl – benzyl – ammoniumchlorid 15%) one to two hours before planting the treated seedlings.


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1.4 Statistics

Analyses of the variance (ANOVA) of induction and doubling rates were performed using the MIXED procedure of the SAS® 9.1.3 software (SAS Institute Inc., 2004). Replicons were treated as random factor and waxy*QPM donor hybrids, inducer lines and their interaction as fixed effects. Sources of variation and appropriate F ratios (Type III) were applied according to McIntosh (1983). The comparison of means estimates was performed with a Student Newman-Keuls test (t test). Standard deviation, confidence interval and box plots were performed using the MEANS and BOXPLOT procedure of SAS® 9.1.3 software.

1.5 Results

1.5.1 Selection of haploid seeds after induction

1.5.1.1 Visual selection according to anthocyanin pigmentation

After the step of haploid induction, where maternal donors were hand-pollinated by pollen of inducer lines, 2842 induced seeds were harvested and separated into three bulks according to their aleurone and embryo color. The expression of anthocyanin (red crown) varied depending on the maternal genotype; it could be inhibited or over-expressed. Seeds with pigmented embryos (1222 seeds, 43% of the induced seeds) were identified as diploid F₁ hybrids between the donor hybrid and the inducer line and no longer taken into consideration. The main issue was to eliminate the false positives (unpigmented embryo but diploid) present in the two bulks: bulk 1 (1151 seeds, 40% of the induced seeds) of seeds with unpigmented embryo and unpigmented endosperm (also called unpigmented seeds) and bulk 2 (469 seeds, 17% of the induced seeds) of unpigmented embryo but pigmented endosperm (so called putative haploid seeds) (Fig 1.1).

Except TH6, four hybrids (CN3, CN35, CN37, and VN33) showed originally yellow and vitreous kernels. After in vivo maternal haploid induction, their progeny segregated with regard to kernel color from dark yellow to white (Fig 1.6). The hybrid TH6 produced only seeds with pigmented endosperm, when induced by the inducer line RWK76, but generated almost 50% of unpigmented seeds when induced by the other two inducer lines (similar to the seed set obtained on the other donor hybrids). For the hybrid CN35, the whole progeny carried the typical “red-crown” aleurone after induction by any of these three inducer lines, indicating the successful induction. The other induced hybrids produced progenies with both, pigmented and
unpigmented endosperm. The proportion of unpigmented seeds (bulk 1) among the progenies of different inducer line and hybrid combinations were high compared to data reported in the literature, ranging from 34.8 to 56.5% (with the exception of CN35 (lower than 2% across the three inducer lines) and TH6*RWK76 (0.0%)) (Table 1.2). Except on CN35, almost half of the induced seeds of each combination carried a unpigmented endosperm. The bulk 1 of induced seeds of hybrid CN35 consisted only in a few unpigmented seeds, among which no haploid seeds were detected by flow cytometry.

For the different hybrids, the rate of unpigmented seeds (relative to the total number of induced seeds) was similar across the three inducer lines, ranging from 29.4 to 39.7% (Table 1.2). However, the proportion of putative haploids in the induced progeny (pigmented endosperm and unpigmented embryo) varied with regard to the inducer line used and to the donor genotype from 5.7% up to 45.1%.

Table 1.2. Rate (%) of unpigmented seeds (bulk1) and of putative haploids seeds (bulk2) among the induced seeds progeny of five waxy*QPM hybrids, CN3, CN35, CN37, VN33 and TH6 after pollination by three different inducer lines, RWS, RWK76 and RWK76RWS. Rates (%) are relative to the total number of induced seeds of a specific combination of donor hybrid and inducer line.

<table>
<thead>
<tr>
<th>Hybrid</th>
<th>Bulk 1 (unpigmented seeds)</th>
<th>Bulk 2 (putative haploid seeds)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RWS</td>
<td>RWK76</td>
</tr>
<tr>
<td>CN3</td>
<td>53.5</td>
<td>48.7</td>
</tr>
<tr>
<td>CN35</td>
<td>1.9</td>
<td>0.0</td>
</tr>
<tr>
<td>CN37</td>
<td>51.7</td>
<td>53.4</td>
</tr>
<tr>
<td>VN33</td>
<td>34.8</td>
<td>44.7</td>
</tr>
<tr>
<td>TH6</td>
<td>56.5</td>
<td>0.0</td>
</tr>
<tr>
<td>Mean</td>
<td>39.7±19.9§</td>
<td>29.4±23.6</td>
</tr>
</tbody>
</table>

§, confidence interval of the average values at the 0.05 probability level are given in italic and bold
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Table 1.3. ANOVA for the effect of different inducer line and waxy*QPM hybrid combinations on the amount of unpigmented (Bulk 1) and putative haploid (Bulk 2) seeds among the progeny.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>DF</th>
<th>Mean Square Bulk 1</th>
<th>Mean Square Bulk 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fixed effects</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inducer</td>
<td>2</td>
<td>238.3**</td>
<td>213.4***</td>
</tr>
<tr>
<td>Hybrid</td>
<td>4</td>
<td>2522.5***</td>
<td>347.4***</td>
</tr>
<tr>
<td>Hybrid x Inducer</td>
<td>8</td>
<td>289.8**</td>
<td>186.7***</td>
</tr>
<tr>
<td>Random effects</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rep</td>
<td>14</td>
<td>34.9</td>
<td>10.8</td>
</tr>
<tr>
<td>Residual</td>
<td>0</td>
<td>1.1E-12</td>
<td>1.1E-12</td>
</tr>
</tbody>
</table>

**, significant at the 0.010 probability level
***, significant at the 0.001 probability level

Fig 1.3. Proportion of bulk 1 (unpigmented seeds) and bulk 2 (putative haploid seeds) in the induced seeds of five waxy*QPM hybrids. The box defines the first quartile (Q1) and the third quartile (Q3). The horizontal line inside the box represents the median. The two outer lines are the min and max values.

The combination inducer line and donor hybrid significantly impacted the proportion of unpigmented and putative haploid seeds among the progeny (Table 1.3). Moreover, the interaction between the inducer line and the maternal donor was significant with regard to the
rate of putative haploid induction and may play an important role in the expression of anthocyanin pigmentation.

The rates of putative haploids among the progenies induced with the inducer lines RWS and RWK76 (21.1 and 19.8% in average, respectively) were significantly higher ($P<0.05$) than those observed with the inducer line RWK76RWS (12.5%) for the same maternal donor plant material. Among the five hybrids induced with RWS, CN37 had the highest proportion of putative haploid seeds among the progeny (42.4%).

### 1.5.1.2 Haploid selection by flow cytometry

The ploidy level of seeds of the two bulks, unpigmented seeds (bulk 1) and putative haploid seeds (bulk 2), was assessed by flow cytometry (FCM). This method permitted first to detect false positives among the putative haploids, second, to detect haploid seeds in bulk 1, which indicated the existence of genetic factors that may interfere/inhibit the expression of anthocyanin in specific combinations of waxy*QPM plant material. A quite high number of real haploid seeds (between 2.8% and 10.2% of the total number of seeds of bulk 1) was found among the unpigmented seeds and almost half of the putative haploid seeds (bulk 2) were identified as diploid (Table 1.4).

#### Table 1.4. Proportion of real haploid seeds (%) in the bulk1 of unpigmented seeds, bulk2 of putative haploid seeds and real haploid induction rate (%) (relative to the total number of induced seeds) as indicated by flow cytometry.

<table>
<thead>
<tr>
<th>Hybrid</th>
<th>Bulk1 (unpigmented seeds)</th>
<th>Bulk2 (putative haploid seeds)</th>
<th>Real haploid induction rate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RWS</td>
<td>RWK76</td>
<td>RWK76-RWS</td>
</tr>
<tr>
<td>CN3</td>
<td>7.5</td>
<td>5.2</td>
<td>6.2</td>
</tr>
<tr>
<td>CN35</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>CN37</td>
<td>10.2</td>
<td>4.9</td>
<td>3.8</td>
</tr>
<tr>
<td>VN33</td>
<td>3.0</td>
<td>4.1</td>
<td>2.8</td>
</tr>
<tr>
<td>TH6</td>
<td>7.0</td>
<td>0.0</td>
<td>4.3</td>
</tr>
<tr>
<td>Mean</td>
<td>5.5±3.5</td>
<td>2.8±2.3</td>
<td>3.4±2.02</td>
</tr>
</tbody>
</table>

§, value (in column) followed by the same letter are not different at the 0.05 probability level

#, confidence interval of the average values at the 0.05 probability level are given in italic and bold
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The proportion of real haploid seeds in the two bulks strongly varied depending on the waxy*QPM donor hybrid but the total real haploid rates were similar for the three inducer lines (Table 1.4). False positives seeds occurred in bulk 2 (putative haploid seeds) of hybrid CN35 as well even though none of its unpigmented induced seeds (bulk 1) were detected as haploid (Table 1.2).

![Bar chart showing the proportion of real haploid seeds in bulk 1 (unpigmented seeds) and bulk 2 (putative haploid seeds) for each waxy*QPM hybrid after induction by three inducer lines. The box defines the first quartile (Q1) and the third quartile (Q3). The horizontal line inside the box represents the median.](image)

Fig 1.4. Proportion of real haploid seeds in bulk1 (unpigmented seeds) and bulk2 (putative haploid seeds) for each waxy*QPM hybrid after induction by three inducer lines. The box defines the first quartile (Q1) and the third quartile (Q3). The horizontal line inside the box represents the median.

**Table 1.5. ANOVA for the effect of the waxy*QPM hybrid (Hybrid) on the proportion of haploid seeds in bulk 1 (unpigmented seeds) and bulk 2 (putative haploids seeds). The inducer line (inducer) effect was treated as random factor.**

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>DF</th>
<th>Mean square</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Bulk 1</td>
</tr>
<tr>
<td>Fixed effects</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hybrid</td>
<td>4</td>
<td>20.3*</td>
</tr>
<tr>
<td>Random effect</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inducer</td>
<td>2</td>
<td>10.1 NS</td>
</tr>
<tr>
<td>Residual</td>
<td>8</td>
<td>3.9</td>
</tr>
</tbody>
</table>

*, significant at the 0.05 probability level

NS, non-significant
1.5.2 Chromosome doubling

1.5.2.1 Recovery after treatment

Mortality rate due to different colchicine treatments was evaluated in a parallel experiment on diploid seeds from each variety. The control situation (Ex.5) showed that the immersion of the coleoptiles for 12h in a liquid solution did not affect the latter plant growth (Table 1.6). As well, for the five different Chinese waxy*QPM hybrids tested in these experiment, the punctual injection of colchicine at the 3rd-4th leaf stage (Ex.4) was not lethal for the young plants (Table 1.6 and Fig 1.5). Under these experimental conditions the seedlings of these five hybrids were able to recover well after the different colchicine treatments.

The genetic background did not significantly influence the mortality rate, which was usually lower than 20% under experimental conditions Ex.1, Ex.2 and Ex.3 for all varieties (Table 1.6). However no colchicine treatment was harmless and different treatment conditions significantly influenced the number of survival plants (Table 1.7). A treatment of longer duration on the shoot (Ex. 3) and the root system (Ex.2) led to higher mortality (P<0.001) than the original method (Ex.1), where the whole coleoptile was treated.

Table 1.6. Mortality and doubling rates (%) (relative to the total number of seeds treated) after different chromosome doubling treatments of seedlings derived from five Chinese waxy*QPM hybrids.

<table>
<thead>
<tr>
<th>Hybrid</th>
<th>Mortality rate (%)</th>
<th>Doubling rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ex.1</td>
<td>Ex.2</td>
</tr>
<tr>
<td>CN3</td>
<td>12.2</td>
<td>13.3</td>
</tr>
<tr>
<td>CN35</td>
<td>6.7</td>
<td>13.3</td>
</tr>
<tr>
<td>CN37</td>
<td>10.0</td>
<td>10.0</td>
</tr>
<tr>
<td>VN33</td>
<td>16.7</td>
<td>23.3</td>
</tr>
<tr>
<td>TH6</td>
<td>16.7</td>
<td>20.0</td>
</tr>
</tbody>
</table>

1.5.2.2 Doubling rate
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The chromosome doubling rate is crucial for determining the success of the process of producing doubled haploids. The results obtained in Ex.5 (control treatment with water) showed that no spontaneous chromosome doubling happened in these plants (Table 1.6 and Fig 1.5). The injection treatment (Ex.4) did not induce any doubling event either and was found to be an inappropriate method for doubling the chromosome set of these plant materials.

Table 1.7. ANOVA for the effect of different treatments on the mortality and doubling rates of seeds derived from five waxy*QPM hybrids

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>DF</th>
<th>Mortality rate</th>
<th>Doubling rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fixed effects</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hybrid</td>
<td>4</td>
<td>189.1**</td>
<td>52.2NS</td>
</tr>
<tr>
<td>Experiment</td>
<td>4</td>
<td>591.3***</td>
<td>16530.0***</td>
</tr>
<tr>
<td>Hybrid*Experiment</td>
<td>16</td>
<td>28.4NS</td>
<td>147.4NS</td>
</tr>
<tr>
<td>Random effects</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rep</td>
<td>2</td>
<td>383.1***</td>
<td>756.3**</td>
</tr>
<tr>
<td>Residual</td>
<td>28</td>
<td>34.7</td>
<td>1.1E−12</td>
</tr>
</tbody>
</table>

NS, non-significant
** , significant at the 0.010 probability level
***, significant at the 0.001 probability level

Fig1.5. Mortality and doubling rates of the different colchicine treatments. The box defines the first quartile (Q1) and the third quartile (Q3). The horizontal line inside the box presents the median. The two outer lines are the min and max values.
Fig 1.6. Cobs of five waxy*QPM hybrids (in lines), CN3, CN35, CN37, VN33 and TH6, after maternal haploid induction with three different inducer lines (in column), RWS, RWK76 and RWK76RWS.
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The best doubling rates were achieved with the methods Ex.1 and Ex.2 (Table 1.6). Ex.1, where the whole coleoptile was immerged into the colchicine solution, showed a high doubling rate but also a high number of abnormal and mosaic plants, which needed a longer time to recover (data not shown). Ex.2, where only the roots of the seedlings were immerged in the colchicine solution, resulted usually in a lower doubling rate than Ex.1 (50% in average across five genotypes) but these plants were more vigorous and recovered faster than in treatments Ex.1 and Ex.3.

1.6 Discussion

1.6.1 Selection of haploid seeds among the induced seeds

*In vivo* gynogenesis to produce DH mostly relies on the visual marker *R1-nj* for anthocyanin pigmentation. In this plant material, the appearance of unpigmented induced seeds illustrated that the expression of anthocyanin pigmentation may result from a genetic interaction between the inducer line (responsible for the pigmentation) and related genes of the maternal donor hybrid. In this study, this interaction was probably responsible for the presence of many false positive seeds in the bulk2 of putative haploids.

A high proportion of unpigmented seeds was observed, when hybrids between subtropical and tropical waxy maize and QPM were used as maternal donors for generating haploids after having been pollinated by inducer lines, which should not occur in theory. Especially, most of the kernels with unpigmented endosperm had unpigmented embryo as well; very few of them exhibited colored embryos. The ploidy level of all unpigmented and putative haploids was controlled by flow cytometry in order to identify the real haploids. The presence of 2.8% to 10.2% of real haploids among the set of unpigmented seeds was assessed (except for the hybrid CN35). Besides, a relatively high amount of false positives, i.e. diploid seeds, was also detected in the bulk of putative haploids. The number of real haploids eventually confirmed by FCM attested the necessity of combining these two selection methods for an effective selection of haploid seeds as a first step toward DH production with this plant material.

A relatively high rate of false positives was reported in other studies, too. By applying the method of producing DH derived from European flint plant material, Röber et al. (2005) found an unacceptable high number of misclassification among putative haploid seeds after induction with RWS. Despite the failure in anthocyanin pigmentation of the aleurone of induced
seeds, haploids could still be distinguished from diploid seedlings later, according to the red coloration of the stem due to the sun-independent gene carried by the inducer line. Belicuas et al. (2007) studied paternal haploid induction with the inducer line W23 on tropical donor material and achieved from 0 to 51% of putative haploid seeds, depending on the paternal genetic. Eventually, only four among 462 putative haploid seeds (0.9%) were identified as real haploids by the mean of SSR markers, and eventually confirmed by flow cytometry and chromosome counting.

Among the subtropical/tropical waxy*QPM maternal donors used in this project, the hybrid CN35 was the only one on which the standard protocol of selection on the manner of anthocyanin pigmentation could be followed. All seeds derived from this hybrid showed a pigmented endosperm after haploid induction with any inducer line. Almost 30% of its putative haploid seeds were found to be diploid, and thus false positives. However, this rate remains acceptable.

The high proportion of unpigmented seeds (bulk 1) as well as the detection by FCM of haploids among these seeds illustrated that the anthocyanin pigmentation was not reliable/sufficient method for detecting haploid seeds derived from subtropical/tropical flint plant material. Our results confirmed that the anthocyanin pigmentation and, as a consequence, the reliability of the visual selection method, was barely dependent on the maternal donor genetic. In general, the proportion of real haploids obtained after haploid induction with the three inducer lines ranged from 9.6 % to 13.3% (Table 1.4), which was higher than the average of 8% usually reported for the inducer line RWS (Röber et al., 2005). Even though the extra-effort for selecting haploid seeds among the induced progeny to some extent disqualifies the technique for a fast routine application, the relatively high proportion of real haploid seeds remains promising and encouraging for implementing inducer lines on subtropical and tropical plant material in order to generate DH for maize breeding.

1.6.2 How waxy*QPM maternal donor genetic could interfere with or inhibit anthocyanin pigmentation.

Anthocyanin pigmentation of the maize aleurone is a complex system that is environment and genetic dependent (Cone, 2007). The gene Cl plays a regulatory role in the production of anthocyanin pigments. Beside Cl, a couple of other genes like Rl, Bzl were reported to be interacting with Cl to form anthocyanin pigments (Cone, 2007). The complex
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regulation of anthocyanin production system seems dependent on two other genes/alleles, the gene $C1^I$, which acts as an inhibitor of $C1$ and the recessive gene $c1$, which induces no pigmentation of the maize aleurone.

The three inducer lines used in our study carry the gene $R1-nj$ for red crown endosperm and red embryo. Thus, in the ideal case, a red endosperm is expected for the whole progeny resulting from the cross between the maternal donor hybrid and the inducer line. According to the frequency of almost 50% of unpigmented seeds among the induced seed (Table 1.2), one could hypothesize that maternal donors may carry a heterozygous gene ($C1^Ic1$) which may inhibit the expression of the paternal allele $R1-nj$. After pollination by the inducer lines ($C1C1$), the progeny segregates with regard to the expression of anthocyanin pigmentation (Table 1.8).

The exception of the hybrid CN35 could be explained by a homozygous $c1c1$ form of this gene in the maternal donor hybrid CN35, which would finally lead to induced kernels with pigmented endosperm ($C1c1c1$) (Table 1.8). The waxy*QPM hybrid TH6 did not produce unpigmented seeds after having been pollinated by RWK76, which could indicate the presence of a homozygous $c1c1$ maternal donor but this maternal donor still produced two different kinds of seeds after having been induced by the two other haploid inducer lines, which gives evidence for a rather heterozygous $C1^Ic1$ maternal genetic. In such a situation, we could only suspect a case of impurity in the maternal donor seed set or the presence of other factors that may play a role as well in the expression of anthocyanin pigmentation.

Table 1.8. Possible combinations of three alleles $C1$, $C1^I$ and $c1$ in the parental lines and eventually in the endosperm and embryo of induced seeds with the related effect on the pigmentation ($P$, pigmented or $N-P$, unpigmented) of the triploid endosperm and the embryo (haploid or diploid).

<table>
<thead>
<tr>
<th>Inducer lines</th>
<th>Maternal donors</th>
<th>Endosperm</th>
<th>Induced seeds</th>
<th>Embryo</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C1C1$</td>
<td>$Cl^I c1$</td>
<td>$N-P$</td>
<td>$Cl^I C1c1$</td>
<td>$P$</td>
</tr>
<tr>
<td>$c1c1^b$</td>
<td>$N-P$</td>
<td>$P$</td>
<td>$cl$</td>
<td>$N-P$</td>
</tr>
</tbody>
</table>

§, possible situation of CN35
Besides, *modifier* genes for \( o2 \) present in QPM are other factors that may alter the “red crown” coloration of the aleurone (Becraft and Asuncion-Crabb, 2000; Lopes, M. A. and Larkins, 1991) and result in alternative crowns like only a red dot on the aleurone (Fig 1.6).

### 1.6.3 Chromosome doubling

Chromosome doubling is the most important and the most investigated step of the process of producing doubled haploids. Successful doubling of chromosomes depends not only on the method applied, but also on the genetic of the plants treated (Geiger, 2009; Kasha, 2005; Segui-Simarro and Nuez, 2008). Several chemicals and pretreatment conditions have been reported as available methods to double the chromosome set. Colchicine is the most common chemical used for chromosome doubling in many plants, including maize. However, with the special biologic structure of maize, where meristems are completely covered by leaves and sheaths, the success of colchicine application was still limited. By applying Deimling’s method (1997), we expected to generate numerous plants with a doubled set of chromosomes. The mortality and doubling rates observed after treatment of plant material derived from five subtropical and tropical waxy*QPM hybrids were acceptable. The plant’s recovery ability as well as the doubling rate depended on the treatment applied (Table 1.7). Thus, finding the optimal chromosome doubling method for haploid seedlings would probably substantially step up the implementation of DH technique in practical maize breeding of subtropical/tropical plant material as well.

Since colchicine is well known as one of the most hazardous chemical for plants, we tried to optimize Deimling’s method for improving the process of chromosome doubling and increasing the survival rate. Alternative methods as well as the method consisting in injecting directly colchicine into the meristem of young plants were expected to be more effective than the original protocol. However, no alternative treatment led to a higher success in chromosome doubling than the original method. Furthermore, as diploid seedlings have been used in this experiment instead of haploids, they should even have had a higher fitness. It is noteworthy that the survival and doubling rates observed in this experiment might be higher than the ones to be expected when applying similar treatments to haploid seedlings.
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1.7 Conclusion

As waxy*QPM hybrids may carry anthocyanin inhibitor factor, misclassification of seeds were observed when selecting the haploid seeds according to anthocyanin pigmentation as it is usually done. The assessment of the ploidy level by flow cytometry was crucial to identify real haploid seeds. To optimize the selection process, pre-selection on the mean of anthocyanin pigmentation is recommended to select all seeds with unpigmented embryo. The real haploids can thereafter be identified by testing the ploidy level of the pre-selected seeds by FCM. In this study, high haploid induction rates were achieved when subtropical/tropical waxy*QPM were induced by modern European inducer lines, which is very encouraging for further implementation of inducer lines on this plant material as well.

No improvement of the efficiency of chromosome doubling and/or plant fitness could be observed when alternatives of Deimling’s method for chromosome doubling were applied. Thus, following this method for further experiments with this kind of subtropical/tropical plant material is recommended. However, as the results were assessed on diploid seedlings, the rates of mortality and chromosome doubling when treating haploid seedling may be slightly different. The proportion of induced haploid seeds derived from five subtropical/tropical waxy*QPM hybrids and their chromosome doubling rate were comparable to those obtained with European maize germplasms, which is promising for the implementation of DH production on exotic material in the future.
Chapter 2

Application of in vivo gynogenesis to subtropical and tropical waxy*QPM hybrids

Abstract

Waxy maize, first found in China in the 1900’s, plays a very important role toward food security in China and other countries in South East Asia as a food staple for large communities and populations. Since waxy maize is of poor nutritional value, the idea of introgressing the quality protein maize (QPM) trait in order to enhance its protein quality and thus its nutritional value arose. In this chapter, we used the technique of inducing doubled haploids (DH) derived from waxy*QPM hybrids with the aim to generate new waxy maize inbred lines with an improved protein quality. Subtropical and tropical waxy*QPM hybrids (CN3, CN4, CN6, CN35 and CN37) were used as maternal donors and induced by modern European inducer lines (RWK76 and RWK76RWS). The application of modern European inducer lines to subtropical and tropical maternal donors was not optimal and especially the expression of anthocyanin pigmentation involved in the selection of haploid seeds among the progeny was affected by the maternal background. However, the final haploid induction rates eventually detected by flow cytometry ranged from 3.1% to 13.7% across five Chinese waxy*QPM hybrids. Combined to chromosome doubling rates of up to 54%, these results illustrate the potential of using the technique of producing DH on subtropical and tropical plant material as well.

2.1 Introduction

2.1.1 Maize development in Asia

Nowadays, maize plays a very important role in Asian agricultural systems according to its diversity in usage for both animal feeding and human consumption. Waxy maize is not only the main staple food for minority ethnic groups in margin areas of many South East Asian (SEA) countries, it is consumed as well as vegetable in central and low-land regions. Besides, normal maize is also used as animal feed.

In the last 40 years in Asia, agriculture in general and maize production in particular has developed very fast. Since 1960, maize yield and global production increased steadily (Fig 2.1). Considering hunger and shortage of food availability, some countries in SEA like Vietnam, Philippines or Indonesia mainly succeeded in reducing hunger and do even export many food
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productions. However, in the margin areas of SEA, where the communication systems are very limited and the water and soil quality is deficient, people, especially minority ethnic groups, are still suffering from hunger and malnutrition. Beside that, the decrease in cultivated lands, the population booming and climate change forced local maize breeders to consider new technologies and genetic sources in order to ensure food security in the future and to improve the nutritional quality of their products for all populations.

![Graph showing the evolution of maize cultivation in Asia since 1960](image)

**Fig 2.1. Evolution of maize cultivation in Asia since 1960 (FAO-STAT, 2009)**

### 2.1.2 Waxy and quality protein maize breeding programs

In Asia, three different types of maize are currently cultivated: normal maize (also called field maize), waxy maize and quality protein maize (QPM). Waxy and QPM are specialty maize varieties that are of high interest for SEA breeders.

Waxy maize is mainly grown for human consumption in up-land areas and in a smaller extent in central low-land. Waxy maize differentiates from regular maize in the proportion of amylopectin in its starch, which reaches almost 100% (Coe et al., 1988; Fergason, 2001; Weatherwax, 1922). Waxy trait in maize is controlled by a recessive $wx$ allele originating from a mutation in the $Wx$ gene located on chromosome 9 (Fergason, 2001; Nelson, O. E., 1968). It is only expressed in the pollen, embryo sac and endosperm.

High quality protein maize, in which the lysine content of the maize endosperm is nearly doubled, is controlled by the recessive allele $o2$ of the $Opaque2$ gene located on chromosome 7.
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(Babu et al., 2005; Gibbon and Larkins, 2005; Lopes, M. A. and Larkins, 1995; Lopes, M. A. et al., 1995). The high quality protein maize has opaque kernels, less vigorous plants, low yield than wild type maize and therefore was not preferred although its nutritional quality was higher. QPM is an improvement of standard o2 maize with regard to endosperm and agronomic traits. It has vitreous endosperm, higher yield, is more vigorous than the standard o2 counterpart (Vasal, S.K., 2001). Even though QPM maize contains a little less of lysine and tryptophan than the standard o2 maize, its lysine content is still about 30% higher than in wild type maize.

Conventionally, backcross (BC) and self-pollination are the main ways for breeding new waxy maize lines and QPM is until now breed by hybridizing inbred QPM lines developed at International Maize and Wheat Improvement Center (CIMMYT, Mexico) by recurrent selection. In breeding programs, the development of inbred lines is the stage where the greatest amount of de novo genetic variation is present, created through genetic recombinations that give rise to novel alleles and new allelic variation (Lee and Tracy, 2009). The aim of recurrent selection is to recover the recurrent parent genetic where the new characteristic(s) of interest has been introgressed (Tracy, 2004). In each BC, 50% of the recurrent parent’s alleles are recovered, which means that the development of elite QPM inbred lines by this process is very time and labor-intensive. Besides, unwanted alleles of the donor parent may be conserved due to linkage and epistasis phenomena. The application of molecular markers as selection tools can facilitate the selection of interesting genotypes and so speed up the process.

2.1.3 Double haploid in modern maize breeding

Producing doubled haploids (DH) is not a new method for developing inbred lines but it became more attractive to breeders after in vivo haploid inducer lines were introduced (Geiger, 2009). With this technique, homozygous plants are directly generated out of heterozygous plants or populations in only one life cycle (i.e. around seven months for maize). The DH population can thereafter be screened for relevant traits and directly integer the breeding program as an inbred line. DH can be produced by either paternal or maternal manner (see chapter 1). Currently, most of the DH lines are generated by maternal haploid induction, so-called in vivo gynogenesis. Production of DH is now the main method for creating a homozygous maize line in leading breeding companies like Pioneer (USA) and KWS (Germany). In 2008, Pioneer claimed that their production of doubled haploid lines increased by more than 200 % (company’s advertisement poster). KWS also has a high number of doubled haploid lines in its
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genetic pool (company presentation 2008). However, the application of DH is limited to world-leading seed companies in Europe and the USA and almost not implemented by local Asian breeders. Some developing countries in SEA benefit from the possibility to produce two to three maize generations a year thanks to subtropical/tropical climate, which definitely shortens the time needed to produce an inbred line by conventional breeding compared to temperate regions. Therefore, backcrossing and pedigree breeding remain the main methods for inbred line development in subtropical and tropical regions.

The main objective of this project was to improve the nutritional quality of waxy maize with regard to the protein profile by introgressing the QPM trait. This could help to counter malnutrition of minority ethnic groups in SEA. The application of in vivo haploid induction on subtropical/tropical waxy*QPM hybrids and its possible application for breeding of subtropical and tropical waxy plant material with enhanced protein quality was investigated.

2.2 Materials and methods

2.2.1 Plant material

Five Chinese waxy*QPM hybrids (CN3, CN4, CN6, CN35 and CN37) were used as maternal donors for producing doubled haploids. These hybrids were derived from five Chinese waxy varieties by crossing with QPM inbred lines. The two modern European haploid inducer lines, RWK76 and RWK76RWS (hybrid between RWK76 and RWS, another inducer line commonly used in Europe) used in this study were kindly provided by the University of Hohenheim, Germany. Five haploid populations ETH3, ETH4, ETH6, ETH35 and ETH37 were achieved after induction of CN3, CN4, CN6, CN35 and CN37, respectively.

2.2.2 Haploid induction and selection

Five Chinese waxy*QPM hybrids were cultivated in greenhouse (12h photoperiod, 26°C day temperature, 21°C night temperature) during the winter season 2008. The female flower was isolated and pollinated by pollen of the inducer lines in order to produce haploid seeds. The ears were harvested at physiological maturity and hand-threshed.

Seeds with a white embryo (pigmentation of the endosperm was not considered) were considered as putative haploid seeds. The real ploidy level of these putative haploids was determined by flow cytometry (FCM). The small tip of the first leaf (coleoptile removed) was cut and chopped into small pieces with a razor blade and stained with 2ml DAPI (4′,6-
diamidino-2-phenylindone) staining solution (5μg/ml, Partec GmbH, Germany). After filtration through a nylon membrane (50µm mesh size), the filtrate was analyzed by FCM, at a par gain FL1 (fluorescence) of 420 to 430nm (relative fluorescence - RF). A peak set at 60 and 130FL (corresponding to the growth phase 1 (G1) and growth phase 2 (G2), respectively) represented diploid plant material while a peak set at 30 and 70FL corresponded to haploid plant material (Fig 1.2).

2.2.3 Chromosome doubling

The method of Deimling et al (1997) was followed for doubling the set of chromosomes of the selected haploid seedlings. The entire coleoptiles of seedlings (4 to 5 days after germination) were immersed in a solution of 0.06% colchicine and 0.5% DMSO (dimethylsulfoxide) for 12h. Thereafter, the coleoptiles were washed under tap water for 20min and seedlings were grown in pots under greenhouse conditions. Two to three weeks after treatment, leaf samples of the youngest leaf were collected to control the ploidy level of the treated plants by flow cytometry and thus assess the success of the chromosome doubling.

2.3 Statistical analyses

Analyses of the variance (ANOVA) of the induction and doubling rates were performed using the MIXED procedure of the SAS® 9.1.3 software (SAS Institute Inc., 2004). The replicons were treated as random factor and the maternal donor hybrid as the fixed effect. Sources of variation and appropriate F ratios (Type III) were applied according to McIntosh (1983). The comparison of means estimates was performed with a Student Newman-Keuls test (t test). Standard deviation, confidence interval and box plots were performed using the MEANS and BOXPLOT procedure of SAS® 9.1.3 software.

2.4 Results

2.4.1 Haploid induction

2.4.1.1 Anthocyanin pigmentation and its inhibition

The influence of different inducer lines was not tested in this experiment (See chapter 1). Five different Chinese waxy*QPM hybrids were used as maternal donor genotypes and 2943 induced seeds were produced. 1212 seeds (41.2%) of which exhibited unpigmented embryos (putative haploid seeds) (Table 2.1). With the exception of the hybrid CN35, the four other hybrids
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produced between 46.1 and 54.3% putative haploids seeds (relative to the total number of induced seeds) according to the anthocyanin pigmentation of the embryos. The rate of putative haploids produced depended significantly on the genotype of the maternal donor (Table 2.2). Although a few putative haploid seeds derived from hybrid CN35 were completely unpigmented (unpigmented embryo and unpigmented endosperm), they usually exhibited a unpigmented embryo and pigmented endosperm.

Table 2.1. Putative haploid induction rates (%), proportion of real haploid seeds among the putative haploids (%) (as indicated by flow cytometry) and real haploid induction rates (%) achieved by pollinating five Chinese waxy*QPM hybrids with two inducer lines. The putative haploid and real haploid induction rates were relative to the total number of induced seeds

<table>
<thead>
<tr>
<th>Hybrid</th>
<th>Putative haploid induction rate (%)</th>
<th>Mean</th>
<th>Std Dev</th>
<th>CI</th>
<th>Proportion of real haploid seeds (%)</th>
<th>Mean</th>
<th>Std Dev</th>
<th>CI</th>
<th>Real haploid induction rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CN3</td>
<td>54.3&lt;sup&gt;a&lt;/sup&gt; 2.9</td>
<td>6.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.8</td>
<td>[49.7-59.0]</td>
<td>4.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CN4</td>
<td>46.1&lt;sup&gt;a&lt;/sup&gt; 6.3</td>
<td>10.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.4</td>
<td>[36.1-56.0]</td>
<td>3.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CN6</td>
<td>50.7&lt;sup&gt;a&lt;/sup&gt; 17.0</td>
<td>24.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20.5</td>
<td>[23.6-77.8]</td>
<td>6.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CN35</td>
<td>25.7&lt;sup&gt;b&lt;/sup&gt; 8.5</td>
<td>61.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.9</td>
<td>[12.1-39.2]</td>
<td>13.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CN37</td>
<td>48.9&lt;sup&gt;a&lt;/sup&gt; 7.4</td>
<td>17.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.1</td>
<td>[37.1-60.8]</td>
<td>9.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>45.1±13.4</td>
<td>23.7±14.9</td>
<td>8.2±6.09</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Std Dev, standard deviations are given in bold and italic; CI, confidence interval at the 0.05 probability level; §, means (in column) followed by the same letter are not significantly different at the 0.05 probability level.
2.4.1.2 Haploid induction rate

Many of the putative haploid seedlings were eventually identified as diploid by flow cytometry and appeared to be false positive cases, where anthocyanin pigmentation was inhibited. This resulted in real haploid induction rates that were much lower than first expected. The proportions of real haploids among induced seeds of the five maternal donors assessed by

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**Fig 2.2. Proportion of real haploid seeds among putative haploids derived from five waxy*QPM hybrids.** The box defines the first quartile (Q1) and the third quartile (Q3). The horizontal line inside the box represents the median. The two outer lines are the min and max values.

**Table 2.2. ANOVA for putative and real haploid induction rates (%) (relative to the total number of induced seeds) of five Chinese waxy*QPM hybrids after haploid induction by two inducer lines, RWK76 and RWK76RWS.**

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Putative haploid induction rate</th>
<th>Real haploid induction rate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Df</td>
<td>Mean Square</td>
</tr>
<tr>
<td>Fixed effect</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hybrid</td>
<td>4</td>
<td>509.1**</td>
</tr>
<tr>
<td>Random effects</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rep</td>
<td>3</td>
<td>100.4 NS</td>
</tr>
<tr>
<td>Residual</td>
<td>12</td>
<td>91.2</td>
</tr>
</tbody>
</table>

**, significant at the 0.01 probability level; ***, significant at the 0.001 probability level; NS, non-significant

---

2.4.1.2 Haploid induction rate

Many of the putative haploid seedlings were eventually identified as diploid by flow cytometry and appeared to be false positive cases, where anthocyanin pigmentation was inhibited. This resulted in real haploid induction rates that were much lower than first expected. The proportions of real haploids among induced seeds of the five maternal donors assessed by

---
FCM (Table 2.1) were consistent with those reported by the literature and those found in our first experiment (See chapter 1). In general, less than 30% of putative haploid seeds were confirmed as real haploids. The response of the three hybrids CN3, CN4, CN37 was similar with less than 20% of real haploids among the putative haploid seeds. CN6 exhibited a slightly higher (NS) real haploid rate of 24.9% of the progeny. The proportion of real haploids among the putative haploids was the highest for CN35 ($P<0.05$), where 61.7% of the seeds selected as putative haploids were effectively haploid. The maternal donor genotype was determinant ($P<0.001$) for the number of putative and real haploids generated by the induction process.

The proportion of real haploids relative to the total number of induced seeds is more important for breeders than their proportion among the putative ones in order to evaluate the success of in vivo haploid induction on subtropical/tropical waxy*QPM hybrids. A wide range of haploid induction rates was observed, ranging from 3.1% for CN4 to 13.7% for CN35 (Table 2.1). CN35 was the best responding waxy*QPM hybrid for both, reliability of the selection of haploids according to anthocyanin pigmentation and its high haploid induction rate. The hybrid CN37 as well produced a high number of haploid seeds (9.2%), meanwhile the three other hybrids CN3, CN4, CN6 exhibited lower haploid induction rates, ranging from 3.1% to 6.3%.

2.4.2 Chromosome doubling rate

Since neither spontaneous chromosome doubling occurred in this plant material, nor injecting directly colchicine was effective (See chapter 1), the haploid seedlings selected by FCM, underwent Deimling’s method for chromosome doubling. The survival rates were high for seedlings derived from all the five Chinese waxy*QPM hybrids, ranging from 40.0% to up to 80.0% (Table 2.3). Nevertheless, as colchicine is hazardous chemical, haploid seedlings recovered slowly after treatment and exhibited unusual phenotypes (twisted leaves, dwarf plants, slower growth) at the first development stages.

Seedlings of ETH35 survived better than treated haploid seeds derived from the four others maternal donors. However, the genotype of the waxy*QPM hybrid of origin did not significantly impact the survival rate after colchicine treatment (Table 2.4).
Chromosome doubling rate, representing the number of plants with doubled chromosome set relative to the total number of haploid plants treated, was not high (around 30%) in the five populations. With the exception of the haploid population ETH35 with 54.1% haploid seedlings were doubled the chromosome set, it was good-responding to the chromosome doubling step (Table 2.3).

Table 2.3. Survival and doubling rate (%) (relative to the total number of haploid seeds treated) after colchicine treatment of haploid seeds derived from five Chinese waxy*QPM hybrids

<table>
<thead>
<tr>
<th>Haploid population</th>
<th>Survival rate (%)</th>
<th>Doubling rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Std Dev</td>
</tr>
<tr>
<td>ETH3</td>
<td>47.2&lt;sup&gt;ab§&lt;/sup&gt;</td>
<td>42.7</td>
</tr>
<tr>
<td>ETH4</td>
<td>40.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>54.8</td>
</tr>
<tr>
<td>ETH6</td>
<td>63.1&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>29.0</td>
</tr>
<tr>
<td>ETH35</td>
<td>80.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23.9</td>
</tr>
<tr>
<td>ETH37</td>
<td>73.6&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>41.9</td>
</tr>
<tr>
<td>Average</td>
<td>60.5</td>
<td>39.2</td>
</tr>
</tbody>
</table>

Std Dev, standard deviation; CI, confidence interval at the 0.05 probability level; §, means (in column) followed by the same letter column are not significantly different at the 0.05 probability level

Chromosome doubling rate, representing the number of plants with doubled chromosome set relative to the total number of haploid plants treated, was not high (around 30%) in the five populations. With the exception of the haploid population ETH35 with 54.1% haploid seedlings were doubled the chromosome set, it was good-responding to the chromosome doubling step (Table 2.3).

Table 2.4. ANOVA for survival and doubling rate (relative to the number of haploid seeds treated) after colchicine treatment of haploid seeds derived from five Chinese waxy*QPM hybrids

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>DF</th>
<th>Survival rate</th>
<th>Doubling rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fixed effect</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hybrid</td>
<td>4</td>
<td>1693.7NS</td>
<td>455.2NS</td>
</tr>
<tr>
<td>Random effects</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rep</td>
<td>6</td>
<td>3719.1**</td>
<td>821.0NS</td>
</tr>
<tr>
<td>Residual</td>
<td>17</td>
<td>775.8</td>
<td>1132.3</td>
</tr>
</tbody>
</table>

**, significant at 0.01 probability level; NS, not significant
Considering as well the survival rate, around 50.0% of the plants that survived eventually had a diploid chromosome set. Again, seeds derived from CN35 exhibited the best response to chromosome doubling by colchicine treatment with a doubling rate of almost 55.0%.

![Fig 2.3. Survival rates and doubling rates (relative to the total number of haploid seeds treated) after colchicine treatment of haploid seeds derived from five Chinese waxy*QPM hybrids. The box defines the first quartile \( Q_1 \) and the third quartile \( Q_3 \). The horizontal line inside the box represents the median. The two outer lines are the min and max values.]

Fig 2.3. Survival rates and doubling rates (relative to the total number of haploid seeds treated) after colchicine treatment of haploid seeds derived from five Chinese waxy*QPM hybrids. The box defines the first quartile \( Q_1 \) and the third quartile \( Q_3 \). The horizontal line inside the box represents the median. The two outer lines are the min and max values.

Considering as well the survival rate, around 50.0% of the plants that survived eventually had a diploid chromosome set. Again, seeds derived from CN35 exhibited the best response to chromosome doubling by colchicine treatment with a doubling rate of almost 55.0%.

![Fig 2.4. Chromosome doubling and fertility rate of haploid, respectively DH, plants (relative to the total number of haploid seeds treated) of five haploid populations derived from Chinese waxy*QPM hybrids]

Fig 2.4. Chromosome doubling and fertility rate of haploid, respectively DH, plants (relative to the total number of haploid seeds treated) of five haploid populations derived from Chinese waxy*QPM hybrids.
Nevertheless, this higher rate did not statistically differ from that of haploid seeds derived from the four other hybrids. The main purpose of chromosome doubling is to produce homozygous diploid plants that can be multiplied by self-pollination. Therefore the amount of seeds produced by these DH plants is crucial for the evaluation of the success of the whole process.

In general, the fertility rate of DH plants was low; depending on the waxy*QPM hybrid of origin, it ranged from 13.6% for DH of the haploid population ETH35 to 33.0% for those of ETH4 (Fig 2.4.). Doubled haploid plants of ETH37 had a relatively good seed set as well (26.5%).

2.5 Discussion

2.5.1 Haploid seeds selection

Selection of haploids after induction was not reliable on seeds originating from these maternal donor hybrids since the expression of anthocyanin pigmentation was apparently partly inhibited (See chapter 1). Because subtropical and tropical waxy (and therefore waxy*QPM hybrids as well) often carry gene(s) for stem coloration, their haploid progeny could not be selected with the additional red-stem trait carried by the inducer lines that is supposed to facilitate the selection in cases, where classical selection according to anthocyanin pigmentation was not reliable (See chapter 1).

Non-destructive method of controlling all putative haploids (i.e. seeds with a white embryo) by flow cytometry was necessary to assess the ploidy level. Subsequently, we observed that coleoptiles of haploid seedlings were usually smaller than those of diploids and that their roots were shorter and carried less root hair. These observations corroborated the general consensus and were consistent with the results of Rotarenko (2000) reporting significant differences in ear and plant traits between haploid and diploid plants. However, some diploid seedlings often appeared to be later germinating and slower growing than wild types as well, which did not make it possible to discriminate haploid and diploid seedlings among induced seeds according to phenotypic traits only. Nevertheless, these phenotypic differences may be of interest for speeding up the procedure by screening first putative haploids for morphological traits before entering the step of ploidy control by FCM.

Confirming the findings of chapter 1, anthocyanin pigmentation was not express reliably in waxy*QPM hybrids and putative haploid induction rates (obtain by pure visual selection) of
Chapter 2

induced seeds derived from this plant material were substantially higher than real haploid induction rates (eventually assessed by FCM), especially for seeds of hybrids CN3, CN35 and CN37 (Fig 1.6). Haploid induction rates found in different growing and induction seasons, namely spring summer (chapter 1) and winter spring (here), suggested that the environment may influence the induction rate. In proper season (spring summer) a slightly higher (NS) haploid induction rate was found (Fig 1.6 and Fig 2.4). The efficiency in haploid induction on hybrid CN3 was lower in winter season (4.0%) than in summer (11.2%) meanwhile the induction rate of hybrids CN35 (12.4 and 13.7%) and CN37 (10.6% and 9.2%) were almost the same in the two different seasons.

In addition to the results of chapter 1, this study strongly confirmed the existence of inhibitor(s) of anthocyanin expression in subtropical and tropical waxy*QPM hybrids. Anthocyanin pigmentation was poorly expressed in both, endosperm and embryo, of induced seeds derived from these maternal genotypes because waxy*QPM hybrids were probably carrying the $C1'$ gene, inhibiting the expression of anthocyanin (See chapter 1). Similar cases were reported by experts and breeders (Geiger, personal communication 2010). Belicuas et al. (2007) applied in vivo androgenic haploid induction method to the tropical hybrid BRS1010 and reported as well failures in haploid selection method based on anthocyanin pigmentation: only four plants were haploids out of 462 putative haploid plants selected visually. In addition, Fan et al. (2008) and Tian et al. (2009) reported that Chinese waxy maize was originated or mutated from flint maize meanwhile other reports (Eder and Chalyk, 2002; Röber et al., 2005) showed that European flint maize led to higher misclassification rate when visually selecting the haploids among the induced progeny than other germplasms. These results combined to those of chapter 1 show the possibility of anthocyanin inhibition in Asian/SEA flint plant material when applying in vivo DH technique in breeding. Nevertheless, the high haploid induction rates eventually assessed by FCM in our experiment were promising and illustrated the high potential of applying in vivo gynogenesis to subtropical/tropical waxy and/or QPM varieties.

Despite the great benefit of DH lines and the interest of local breeders, the application of in vivo haploid production in Asia in general and SEA in particular is still very limited since it is difficult for local breeding companies to access inducer lines for commercial breeding. So far we know, this study was the first implementation of DH technique on Asian subtropical/tropical waxy maize.
2.5.2 Chromosome doubling

Chromosome doubling by colchicine treatment is the most difficult step in the process of producing DH not only because of high mortality rates but also because of low doubling rates. Although colchicine is known as hazardous chemical for both, environment and living organisms, its application in the procedure of plant DH production keeps growing as no other chemical could compete with it in term of chromosome doubling efficiency. In our study, Deimling’s method appeared to be the proper method for chromosome doubling as it is easily handling and relatively efficient (See chapter 1); with this method, chromosome sets of 27.8% to 54.1% of the haploid seedlings derived from waxy*QPM maternal donor hybrids were doubled. This rate was relatively acceptable compared to those achieved in other DH experiments like that of Gayen et al. (1994), where the immersion of the root system of maize haploid seedlings in the colchicine solution for 12h at 18°C, led to a doubling rate of 18.2%. Another benefit of Deimling’s method is that the seedlings germinated for the first ploidy control by FCM can directly enter the chromosome doubling step without any additional procedure.

Treated plants were usually very weak and very sensitive to any kind of pest and disease. When growing under greenhouse conditions, fungi infestations related to high soil humidity were the most problematic. Besides, the long anthesis silking interval (ASI), already observed on waxy*QPM donor hybrids, were very long for DH derived from this material too and thus, may represent the main bottleneck in applying this method routinely. Long ASI limited substantially the reproductive capacity of the DH, which often produced only very low seed sets after selfing or were even impossible to be selfed. Since tropical maize is often sensitive to photoperiod and temperature in the early vegetative stages, changing the early growing conditions (to short day conditions with higher temperatures) may permit to synchronize the male and female flowerings of the DH (Gouesnard et al., 2002).

The technique of chromosome doubling remains the main obstacle for using in vivo haploid induction in exotic waxy and QPM varieties. However, breeding companies have intensively increased the effort on finding alternative chemicals for chromosome doubling, which are much less harmful and more efficient in term of chromosome doubling rate and fertility rate of the DH. This research was successful in the last years (Geiger, personal communication 2010) and the implementation of DH techniques for developing inbred lines is steadily growing in international maize breeding companies. In order to increase breeders’
interest in applying DH technique in subtropical/tropical maize breeding for human consumption, the implementation of alternative chemicals would probably be necessary.

2.6 Conclusion

Doubled haploids in general, and the technique of *in vivo* haploid induction in particular, is a method of high potential in modern maize breeding. It becomes more and more important step in breeding for European and US maize. This study was so far the first application of *in vivo* haploid induction on subtropical and tropical waxy and quality protein maize. Although the use of anthocyanin pigmentation for selecting the haploids after gynogenesis was not reliable because waxy*QPM* plant material seems to carry inhibitor genes, eventually high haploid induction rates reached in this project may encourage breeders to go further with DH technique on this type of plant material as well. In this study, doubled haploid lines derived from different waxy*QPM* donor hybrids were achieved. The next steps will be to select the double recessive *wx-o2* genotypes among the DH and to investigate their quality.
Chapter 3

Marker assisted selection for combining waxy and high protein quality in maize

Abstract

Waxy maize is not only used as vegetable food in Asia, but also as staple food by many ethnic minority groups in South East Asia (SEA). The deficiency in lysine and tryptophan, the two most important amino acids for human nutrition, is the main disadvantage of utilizing waxy maize for human consumption. In order to improve the protein quality of waxy maize, introgression into subtropical waxy (wx) maize background of the opaque 2 (o2) mutation of quality protein maize (QPM), responsible for high contents in lysine and tryptophan, was performed by using the technique of producing doubled haploids (DH) derived from heterozygous subtropical/tropical waxy*QPM hybrids. In this study, we focused on selecting double recessive wx-o2 plants among the DH progenies derived from five different waxy*QPM hybrids (CN3, CN4, CN6, CN35 and CN37) by using marker assisted selection (MAS). Different markers for detecting o2 and wx alleles were evaluated and the markers phi112 and W4, respectively, were identified as reliable in DH plants derived from these hybrids. The applicability of these molecular markers depended on the genetic background. The wide range of waxy mutations that may be present in this plant material complicated further development of one common universal marker for waxy. Therefore, pollen or endosperm staining with iodine still plays and may still play an important role for selecting waxy genotypes in waxy maize breeding programs in the future. The success of waxy selection with the marker W4 in DH progenies derived from CN35 and CN37 combined to a relatively good response of these maternal donor hybrids to haploid induction and chromosome doubling (See chapters 1 and 2) permitted to achieve an acceptable amount of double recessive wx-o2 DH plants. Eventually, 17.9% and 19.5% of the DH derived from CN35 and CN37, respectively, were confirmed as double recessive wx-o2 genotypes that may be used for further breeding of waxy maize with improved protein quality for SEA and China.

3.1 Introduction

3.1.1 Minority ethnic groups in South East Asia (SEA) and China – Problem of malnutrition and need for solutions
Chapter 3

SEA (except Singapore) and China belong to the group of world developing countries, where cities and capitals concentrate substantially more people than rural and up-land areas. People staying in rural low-land areas are mainly engaged in agricultural activities. The majority of the populations living in marginal areas, often up-lands, belong to minority ethnic groups. These populations usually face problems like limited access to land, water and markets, combined to global poverty and generally low level of education (Huynh et al., 2002). Minority ethnic groups, and especially their infants and children, suffer the most from malnutrition and drastically need to increase their protein uptake. There, rice and waxy maize still remain the staple food by tradition and constraint, and meat is only rarely consumed. In order to improve the protein uptake of minority ethnic groups, the possibility of combining waxy maize and other maize varieties with enhanced protein content, such as quality protein maize (QPM) into double recessive waxy*QPM varieties should ideally suit.

3.1.2 Genetic control of waxy and quality protein maize

3.1.2.1 Waxy maize

Starch of waxy maize differs from that of regular maize as it is constituted of almost 100% of amylopectin, a macromolecule containing both (1-4)- and (1-6)-α-D-glucosidic linkages forming a ramified structure. This endosperm trait is controlled by a single waxy (Wx) gene located on the short arm of chromosome 9, close to the centromere (Coe et al., 1988). The recessive wx allele is a loss-of-function mutant of dominant Wx alleles; several sources corresponding to independent mutations that lead to the waxy trait were reported (Fan et al., 2008; Nelson, O. E., 1968). In some waxy maize breeding programs, specific molecular markers were implemented to identify waxy plants but no universal marker for all mutants was developed so far (Liu et al., 2007). Therefore, the most common method implemented until now for identifying reliably waxy plants is the staining with iodine (I₂/KI) of starch contained in pollen, embryo sac or in the endosperm. In contrast to amylose, the special structure of amylopectin inhibits the formation of coils, where the iodine could be caught, which results in a brown color. In presence of amylose, i.e. in non-waxy plants, iodine remains trapped in the starch structure, which leads to a black or dark blue color.

In cereals, the wx gene is known as an inhibiting factor of the activity of a class of granule bound isoforms of starch synthases (Granule Bound Starch Synthase I, GBSSI) (Hylton et al., 1996; Nelson, O.E. and Rines, 1962). In maize, GBSSI is one of the major isoenzymes
involved in the catalysis of amylose synthesis that occurs in amyloplasts of the endosperm. GBSSI is reported to be highly active in non-waxy maize, but almost absent in waxy maize. Klösgen et al. (1986) described the molecular structure of the Wx gene as a DNA fragment of 3718bp consisting in 14 exons and 13 introns (Fig 3.1).

3.1.2.2 Quality Protein Maize (QPM)

Although the nutritional quality of regular maize is usually low, it remains one of the most important food sources in many developing countries. Two mutants with improved protein quality, the high lysine mutant opaque2 (o2) and the floury2 mutant (fl2), were discovered at the beginning of the twentieth century by Emerson et al. (1935), but their nutritional significance was only highlighted 30 years later by Mertz et al. (1964). The opaque2 mutation impacts the amino acid composition of maize grains by nearly doubling the lysine content of the endosperm, which significantly increases its nutritional value.

In o2 maize, the increase in lysine but also in tryptophan, another essential amino acid, results from the alteration of the relative amounts of different protein fractions constituting the maize endosperm proteins. The zein or prolamine fraction, representing originally 60% of the proteins of the endosperm, is substantially reduced, thus increasing the relative part of the other protein fractions (Gibbon and Larkins, 2005). Since zein is practically devoid of lysine, while the other fractions are rich in this amino acid, the overall amino acid profile of the endosperm shows an increase in lysine. No new proteins are formed and the composition per se of the different protein fractions remains unaffected; the increase in protein quality is only due to the reduction in zein fraction compensated by the increase in other protein fractions.

Furthermore, the o2 mutation leads to negative pleiotropic effects such as lower yields and soft and chalky kernels that are more susceptible to pests and diseases (Vasal, S.K., 2001). In order to overcome the defects of o2 maize, various methods were explored with more or less success. To counter the soft and opaque endosperm of o2 maize, double mutants of o2 and other endosperm mutants were developed. Two combinations, opaque2-floury2 (o2o2fl2fl2) and sugary2-opaque2 (su2su2o2o2) (Garwood and Creech, 1972; Glover et al., 1975), still showed a reduction in grain yield compared to wild types and exhibited unsatisfying kernel phenotypes that would require additional selection effort toward more uniformity and resistance to pests and diseases; although the kernels were translucent, their appearance was somewhat dull (Roundy and Glover, 1975). Some o2 germplasms with hard endosperm and better agronomic traits were
developed by the International Maize and Wheat Improvement Center (CIMMYT, Mexico) by introgressing so-called *modifier* genes for *o2*. The lines and varieties combining the recessive *o2* allele and genetic *modifiers* were named quality protein maize (QPM), in opposition to soft endosperm *o2* varieties, called standard *opaque2*. The molecular basis of these *modifier* factors and the mechanisms by which they overcome *o2*-associated traits in QPM are still unknown.

To facilitate the identification of plants that carry the recessive *o2* allele in QPM breeding programs, two specific simple sequence repeat (SSR) markers were developed: the dominant marker phi112, which identifies *O2* alleles in homozygous and heterozygous forms but does not amplify *o2* (Danson et al., 2006; Vivek et al., 2008), and the co-dominant marker phi057 (Vivek et al., 2008), which makes it possible to identify and distinguish the three haplotypes, *o2o2*, *o2O2* and *O2O2*.

### 3.1.3 Maize breeding – Conventional breeding and application marker assisted selection

#### 3.1.3.1 Conventional breeding

For successful maize hybrid breeding programs, the development of elite inbred lines (IL) is crucial. IL development is the stage, where the highest amount of *de novo* genetic variation is present, created through recombination giving rise to novel alleles and new allelic variation (Lee and Tracy, 2009). Conventionally, backcrosses and self-pollinations combined to phenotypic selection are the main ways for developing IL. By conventional backcrossing, the achievement of new IL usually takes at least seven to nine generations. For ensuring global food security in the future, the requirement in new efficient breeding tools is high.

#### 3.1.3.2 Marker assisted selection and its application in maize breeding

Conventional breeding mainly focuses on phenotypic selection and may not be able to distinguish the influence of the environment or of other factors related to growing conditions from the genetic impact. This can be improved by implementing molecular marker assisted selection (MAS) as a direct selection process, where the trait of interest is selected by the mean of a molecular marker that is either directly linked to the gene of interest or to the genome fragment impacting the expression of this trait. MAS allows to select according to the genotype, independently of environmental and epistatic effects. Meanwhile, MAS is approved as an applicable tool that does complement and substantially facilitate conventional breeding and selection techniques (Balding et al., 2003; Ribaut and Hoisington, 1998). In developed
countries, modern breeding methods applying biotechnologies like doubled haploids (DH) induction or genetic transformations are increasingly used in maize breeding programs but in many developing countries their implementation remains still very limited.

This study took place in the frame of a project that aimed to gain double recessive waxy*QPM maize by developing first wx-o2 DH derived from different hybrids between a QPM line and several subtropical/tropical waxy maize. Since these two traits are only clearly detectable at later stages of plant development, specific molecular markers for wx and o2 may be very useful to facilitate the early selection of double recessive wx-o2 genotypes. The applicability of two markers for o2, phi112 and phi057, was investigated on this specific plant material. According to the Maize Genetics and Genomics Database (Lawrence et al., 2008), three primers pairs, phi022, phi027 and phi061, were available for the selection of wx but these markers were not suitable for the plant material used in our study as they were not discriminating confirmed waxy and non-waxy maize lines. Liu et al (2007) designed intragenic markers based on the structure of the Wx gene (1986) and the partial sequence of wx gene (DQ333229) (Miller et al., 2009) and reported two primers pairs, P7P8 and S1S2, that were able to detect either only non-waxy genotypes or only waxy genotypes for several Chinese waxy inbreds, respectively. Fan et al (2008) reported evidence at the molecular level for high allelic variability in the waxy gene of Chinese waxy maize and provided several waxy sequences of inbreds, which can be used for further development of new molecular markers for wx.

The main objective of this study was to evaluate the possible application of MAS on haploid/doubled haploid plant material derived from five heterozygous Chinese waxy*QPM hybrids with the aim to select toward the combined specialty traits, waxy endosperm and high quality protein profile. The reliability of the selection of homozygous recessive o2 genotypes by the mean of the primers pair phi112 and phi057 and of recessive wx with the published primers pairs P7P8, S1S2 and four newly developed markers, W1, W2, W3 and W4 in combination with classical pollen staining with iodine were evaluated.

3.2 Materials and methods

3.2.1 Plant material

Five Chinese heterozygous waxy*QPM hybrids (CN3, CN4, CN6, CN35 and CN37) originating from crosses between Chinese waxy maize (seed parent) and QPM (pollinator) were
produced in 2006 in China and kindly provided by Dr. Wen Renlai (Guangxi Maize Research Institute, China). These hybrids were pollinated with haploid inducer lines (kindly provided by Prof. Geiger H.H., University of Hohenheim, Germany) RWK76 and RWK76RWS, to generate haploid populations ETH3, ETH4, ETH6, ETH35, ETH37, respectively. Young haploid seedlings selected among the induced progeny according to anthocyanin pigmentation and confirmed as haploid by flow cytometry were treated with colchicine to double the chromosome set and thereafter grown in greenhouse for further study and evaluation (See chapter 2). Leaf samples of all surviving plants (haploids/doubled haploids) derived from these five hybrids were sampled and lyophilized for DNA extraction. Due to the lack of information concerning the QPM and waxy maize donor lines of origin, the comparisons where based on the standard QPM lines CML161, T191A-1379P69Q TYF (later abbreviated Q6), the Chinese standard QPM line B102 and the two standard Chinese waxy lines B601 and B602.

3.2.2 Molecular markers selection and evaluation

3.2.2.1 Markers for o2 selection

The dominant SSR marker phi112 and the co-dominant marker phi057 (Table 3.1) were used as selection markers for high protein quality mutation (recessive o2 allele). Phi112 does not amplify the recessive homozygous o2o2, while it amplifies O2 allele(s) and thus detects heterozygous O2o2 and dominant homozygous O2O2 genotypes. Primers pair phi057 is co-dominant and amplifies both alleles with a fragment of 153bp for O2 allele and of around 165bp-170bp for o2 allele. The sequences of these markers were designed upon the O2 gene sequence in maize X15544 (Maddaloni et al., 1989) and are available in the Maize Genetics and Genomics Databank (Lawrence et al., 2008).

3.2.2.2 Marker for wx selection

Primers for waxy selection were based on Klösgen’s non-waxy maize sequence X03935 using primer design software Primer – Blast from the National Center for Biotechnology Information (NCBI) (Johnson et al., 2008), which is based on the software primer3 (Rozen and Skaletsky, 2000). Four primer pairs (from P1P2 to P7P8) (Table 3.1) were designed by Liu et al. (2007) to amplify along the Wx gene (Fig. 3.1).
Three primers pairs (P1P2, P3P4, P5P6), which amplify the Wx gene from intron 1 till exon 14 did not show any difference between non-waxy and waxy genotypes in the plant material considered here. Only primers pair P7P8 was able to discriminate waxy and non-waxy genotypes (by null-amplification and amplification of a fragment of 707bp, respectively), which enabled to use this primers pair for selecting waxy genotypes among this plant material. According to Fan (2008), many Chinese waxy maize (both landraces and inbred lines) were sequenced and published in the Genbank (Miller et al., 2009) for further studies. The alignment between non-waxy gene sequence (X03935) and Chinese waxy landraces and inbred sequences showed a few differences (Fig 3.2). Four additional primer pairs, W1 to W4 (Table 3.1), were designed based on this alignment. W1 targeted the gene fragment comprised between 649bp and 851bp of the non-waxy sequence (Fig 3.1).
Table 3.1. Primers pairs used for O2 and Wx selection with location (bp) (relative to the beginning of the gene of interest) and sequence of the primers, melting temperatures ($T_m$) (°C) and size of the amplification product (bp)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Marker</th>
<th>Position (bp)</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>$T_m$ (°C)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>O2 (X15544)</td>
<td>Phi112</td>
<td>1218-1368</td>
<td>GCC CTG CAG GTT</td>
<td>AGG AGT ACG CTT</td>
<td>57.0</td>
<td>150</td>
</tr>
<tr>
<td></td>
<td>Phi057</td>
<td>3616-3769</td>
<td>CTC ATC AGT GCC</td>
<td>CAG TCG CAA GAA</td>
<td>57.0</td>
<td>153</td>
</tr>
<tr>
<td></td>
<td>P7P8</td>
<td>4054-4760</td>
<td>GGC GTA CGA GGA</td>
<td>CGA AAC AAA CAG</td>
<td>60.0</td>
<td>706</td>
</tr>
<tr>
<td>Wx (X03935)</td>
<td>W1</td>
<td>649-851</td>
<td>GAA TCC TGA CGA</td>
<td>AGA ACA TGG CAG</td>
<td>60.2</td>
<td>202</td>
</tr>
<tr>
<td></td>
<td>W2</td>
<td>4136-4691</td>
<td>AGA GCA GAG CGC</td>
<td>GAA ATT TCA CAA</td>
<td>58.8</td>
<td>555</td>
</tr>
<tr>
<td></td>
<td>W3</td>
<td>4231-4595</td>
<td>CTG CCA AGA ACT</td>
<td>ACT CCA CCA CTA</td>
<td>59.9</td>
<td>364</td>
</tr>
<tr>
<td></td>
<td>W4</td>
<td>4597-4791</td>
<td>AAT AAT CCC TGC</td>
<td>CAG CTT TGG GTG</td>
<td>58.5</td>
<td>194</td>
</tr>
<tr>
<td>Wx</td>
<td>W5</td>
<td>121-517</td>
<td>TTG CGA GTA AAT</td>
<td>CAA AAC TCG GAA</td>
<td>52.0</td>
<td>397</td>
</tr>
</tbody>
</table>

Figure 3.2. Part of sequences alignment of non-waxy (red box) and other Chinese waxy lines (Chinese waxy sequences were obtained from Genebank according to Fan (2008))
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The other three markers bound at the end of the sequence (Fig 3.1); W2 was specific to exon 13 and intron 13, meanwhile W3 and W4 amplified a fragment of exon 14 and of the non-coding flanking region including a part of poly A tail.

3.2.3 DNA isolation and PCR conditions

3.2.3.1 DNA isolation

Twenty milligrams of lyophilized young, healthy leaves were added in a 2ml microcentrifuge tube with two stainless steel balls and ground by swing-mill (Typ MM2, Retsch, Germany) for 4min at 80times/minute. After addition of 600µl of pre-extraction buffer (12.11g Tris-hydroxymethyl-aminomethan, 18.6g NaEDTA, 29.22g NaCl and 2.34mg Na$_2$S$_2$O$_5$, for 1 liter extraction buffer) and 10µl β-mercaptoethanol, the samples were mixed gently and incubated at 95°C for 1h. The supernatant was collected after centrifugation at 11000rpm for 10min at 22°C and incubated with RNase at 37°C for 1h. A solution of 400µl cold (-20°C) commercial iso-propanol or propanol (almost 100%) and 50µl ammonium acetate was added into the tubes. After at least one more hour of incubation at -20°C, the tube was centrifuged at 6000rpm for 10min at 4°C. The precipitate was washed several times with ethanol (75%) and dried (around 1h at 37°C). The DNA solution was obtained by adding 150µl TE (Tris-EDTA) and heating the tubes at 60°C for 5min.

3.2.3.2 PCR conditions

In a final volume of 20µl, 50ng DNA template was mixed with dNTP at 2.5mMol (already in solution with MgCl$_2$), 3.6µl 10x buffer, the two primer solutions at 5pmol each, 0.6U Taq polymerase and 5µl bidistilled water. PCR consisted in an initial denaturation phase at 94°C for 2min, followed by 30 to 35 cycles of denaturation at 94°C for 1min, annealing at 55°C to 65°C depending on the marker (Table 3.1) for 1min, and elongation at 72°C for 2min. A final elongation step of 5min at 72°C was followed by termination of the cycle at 4°C. 12µl of PCR products were loaded for electrophoresis on a 4% agarose gel (standard agarose or high resolution agarose) at 150V for 90 to 180min (depending on the marker).

3.2.4 Pollen staining

According to Pedersen et al (2004), fresh pollen collected on flowering plants was conserved in 0.5ml alcohol (70%) at 4°C. Iodine solution (20µl) (I$_2$/KI - 2.5g KI, 250mg I$_2$, 125ml H$_2$O) was added to 10 µl of well mixed pollen solution on a microscope slide and observed on the mean of 40x magnified lenses on a light microscope.
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3.3 Statistics

Statistical analyses of \(wx\) and \(o2\) frequency were performed with the TTEST procedure of the software SAS®9.1.3 (SAS Institute Inc., 2004). According to the genetic segregation, the frequency hypothesis of 50\% for \(o2\) and \(wx\) and of 25\% for double recessive \(wx\)-\(o2\) genotypes was set.

3.4 Results

3.4.1 Opaque2 selection

Primer phi112 is a dominant marker for \(o2\) (Babu et al., 2005; Danson et al., 2006); in our non-\(o2\) maize, an amplicon of 150bp was amplified by phi112, meanwhile this marker usually did not amplify homozygous \(o2\) genotypes (Fig 3.3). Since the aim was to find a fast, low-cost way of selecting plants that are homozygous for the recessive \(o2\) gene, the marker phi112 and its null-amplification was chosen as the main marker. Primer phi57 was a co-dominant marker and amplified both alleles but the resolution of 4\% agarose gels did not allow to clearly distinguish the two specific fragments that were close together (product of around 155bp for the \(O2\) allele and around 160-170bp for \(o2\) allele) (Fig 3.4). Thus, this marker was not reliable for our non-\(o2\) and \(o2\) genotypes selection process.

Figure 3.3. Homozygous recessive \(o2\) selection with molecular marker phi112. Lanes 1 and 16 are molecular ladder 20bp; Lanes 2 to 6 are heterozygous Chinese waxy*QPM hybrids: CN3, CN4, CN6, CN35 and CN37; Lanes 7 and 8 are Chinese waxy inbred lines, B601 and B602; Lanes 9 and 11 are QPM lines B102, CML161 and Q6; Lanes 12 to 20 are double recessive \(wx\)-\(o2\) plants.
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Chinese QPM inbred line B102 and the two QPM inbred lines of CYMMIT, CML161 and Q6, differed from all other heterozygous hybrids and waxy inbred lines in their response to amplification with the marker phi112. There was no amplification for QPM lines but the same amplicon of around 150bp was detected for the other genotypes (Fig 3.3).

Within doubled haploid populations, different frequencies of homozygous $o_2$ plants were found. Respectively 28.6% and 30.3% of plants of ETH4 and ETH6 exhibited the null-amplification with marker phi112, which indicated homozygous recessive $o_2$ plant material (Table 3.2). Meanwhile, 40.0 to 58.5% of the DH derived from the three other populations, ETH3, ETH35, and ETH37, displayed homozygous recessive $o_2$ haplotypes. However, due to the limited numbers of plants, especially for the two population ETH3 (4 plants) and ETH4 (2 plants), these frequencies were not assessed statistically.

3.4.2 Waxy selection

3.3.2.1 Marker Assisted Selection (MAS)

According to the genetic sequences of different waxy and non-waxy alleles, primers pairs W1, W2 and W3 should amplify fragments of 202, 555 and 364bp, respectively, in non-waxy genotypes. Since in praxis, similar fragments were observed in both non-waxy and waxy genotypes, these three primers pairs were not able to select the genotypes of interest in this plant material (Fig 3.5).

Figure 3.4. Homozygous recessive $o_2$ selection with the molecular markers phi057. Lane 1 to 5 are heterozygous waxy*QPM Chinese hybrids: CN3, CN4, CN6, CN35, and CN37, respectively; Lanes 6 and 7 are Chinese waxy inbred lines, B601 and B602; Lane 9 is molecular ladder 20bp; Lanes 8, 10 and 11 are QPM inbred lines, B102, CML161 and Q6.
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Primers pair P7P8 (Liu et al., 2007), was not satisfying either on our plant material since null-amplifications were not only found in waxy samples as expected, but also in other heterozygous hybrids and QPM lines (Fig 3.6). Since the primers pair S1S2 did not amplify any of the genotypes tested, either waxy or non-waxy (data not show), it was rejected as well).

Figure 3.5. Homozygous recessive wx selection with molecular markers W3 and W4.

W3: Lane 1 is molecular ladder 100bp; Lanes 2 to 4 are heterozygous Chinese waxy*QPM hybrids: CN4, CN6, CN3; Lane 5 is QPM inbred line Q6; Lane 6 is inducer line RWS; Lane 7 and 8 are the Chinese waxy inbred lines B601 and B602; Lane 9 is a negative control; Lane 10 is molecular ladder 100 bp.

W4: lane 1 is molecular ladder 20bp; lane 2 to 6 are heterozygous Chinese waxy*QPM hybrids CN3, CN4, CN6, CN35, CN37; lane 7 is the QPM line B102; Lane 8 is inducer line RWS; Lane 9 and 10 are Chinese waxy inbred lines B601 and B602.

Marker W4 was designed to amplify a fragment of 194bp located at the end of the Wx gene in non-waxy maize. An unexpected specific band of around 800bp was amplified by marker W4 in three hybrids, CN3, CN4 and CN6, while marker W4 amplified a fragment of

Figure 3.6. False positive cases observed with the primers pair P7P8 on Chinese plant material.

ML: Molecular ladder 100bp; Lane 1 to 3 are homozygous recessive o2 genotypes; Lanes 4 to 5 are Chinese waxy inbred lines, B602, B601;Lanes 6 to 7 are heterozygous Chinese waxy*QPM hybrids CN6 and CN3.
around 200bp, i.e. similar to the expected one, in the other two heterozygous hybrids CN35, CN37 and in two QPM inbred lines (Fig. 3.5). Besides, primer W4 did not amplify DNA samples of the two Chinese waxy lines B601 and 602, where no specific band was detected. Therefore, homozygous \( wx \) genotypes could be differentiated from the others by null-amplification with the primers pair W4 that may thus allow identifying waxy genotypes among doubled haploid/haploid plants derived from CN35 and CN37.

Table 3.2. Proportion of homozygous recessive \( o2 \) and \( wx \) genotypes among doubled haploid progenies derived from five heterozygous Chinese waxy*QPM hybrids according to molecular markers phi112 and W4, respectively.

| Population | Nr of plants | Recessive \( o2 \) | | Recessive \( wx \) | | Double recessive \( wx-o2 \) |
|------------|--------------|------------------|-----------------|-----------------|-----------------|
|             | Nr of plants | %                | Nr of plants    | %               | Nr of plants    | %               |
| ETH3        | 10           | 40.0\*           | 2               | 20.0\*          | 1               | 10.0\*          |
| ETH4        | 7            | 28.6\*           | 1               | 14.3\*          | 1               | 14.3\*          |
| ETH6        | 33           | 30.3 NS           | 6               | 18.2 NS         | 0               | 0.0 NS          |
| ETH35       | 41           | 58.5**           | 8               | 19.5 NS         | 8               | 19.5*           |
| ETH37       | 28           | 50.0**           | 7               | 25.0 NS         | 5               | 17.9*           |

NS, non-significant, rejection of the null hypothesis; 
\*, significant at the 0.05 probability level; 
\**, significant at the 0.01 probability level; 
\#, no statistical analysis due to a very low number of plants

A small amount of plants were identified as recessive waxy genotypes (homozygous \( wx \)) by primer W4. Only 15% of the plants of ETH4 showed a null-amplification with W4, which meant that they were waxy. Selected waxy plants frequencies for progenies of the other four populations were similar with around 20% of double recessive \( wx \) among the progeny (Table 3.2). To assess the reliability of results obtained by implementing this newly developed marker, the selected genotypes were tested by the mean of pollen staining.

3.4.2.2 Pollen Staining

In order to control the reliability of the marker selection process for waxy and to improve the pureness of the selected waxy plants, iodine staining of pollen of waxy genotypes selected with W4 was performed. Selected waxy plants of ETH3, ETH4 and ETH6 stained very dark,
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which rendered it impossible to distinguish waxy and non-waxy pollen grains. Therefore, controlling the results of marker selection by pollen staining was not possible for these three populations. Nevertheless, for the two other populations ETH35 and ETH37, pollen staining corroborated the marker results (Fig. 3.7) and confirmed that W4 was a reliable marker for identifying recessive wx genotypes in plant material derived from CN35 and CN37.

Figure 3.7: Maize pollen stained with I2/KI solution. A is pollen of heterozygous Chinese waxy*QPM hybrid CN37; B and C are pollen of two doubled haploid double recessive wx-o2 maize ETH37.1 and ETH37.2 derived from the hybrid CN37.

3.5 Discussion

3.5.1 Quality protein trait selection

In our study, homozygous recessive o2 plants were selected according to the absence of specific amplification band when evaluated with the primers pair phi112. Marker phi112 was developed on the deletion in the promoter region of the o2 gene, which renders it pretty reliable and commonly used for controlling the seed purity of opaque2 varieties (Babu et al., 2005; Xu et al., 2008). In theory, approximately 50% of the haploid (respectively of the doubled haploid) progenies should be homozygous for o2. Among the five populations derived from heterozygous Chinese waxy*QPM hybrids, only the DH populations derived from CN35 and CN37 did statistically exhibit a frequency of 50% of the progeny that carry the recessive o2 allele. The number of individuals screened for populations derived from the two hybrids CN3 and CN4 were too low to allow any statistic assessment of the results (respectively 40.0% and 28.6% of homozygous o2 plants) and questions their reliability. Yang et al. (2004) showed that some lines classified as recessive o2 lines were amplified with the marker phi112 as well; this misamplification may have been caused by the fact that different o2 alleles, due to mutations in
different regions of the gene, may be present in our material. Since the origin of the QPM trait was not clearly identified, the three Chinese waxy*QPM hybrids CN3, CN4 and CN6 may derive from another or several others sources of high quality protein maize; this may explain why they were not reliably detected by marker phi112. In QPM breeding programs, markers phi057 and umc1066 were used as well for discriminating heterozygous and homozygous o2 plants (Babu et al., 2005). These two other markers were not satisfying in our study since the difference in band lengths generated by the primers pair phi057 was not easily visualizable on agarose gels and would require polyacrylamide gels, which are highly toxic and the implementation of which would be more expensive. On the other hand, molecular marker umc1066 was reported as not polymorphic with many CIMMYT QPM inbred lines (Xu et al., 2008), which was also observed in the material of this project (data not show). Thus, no better marker than phi112 could be identified for this plant material in the frame of this project.

3.5.2 Waxy trait selection

The waxy gene is located close to the centromere of chromosome 9 and was recorded as a model for transposable elements (transposons) in maize (McClintock, 1952; Wessler et al., 1987; Wessler and Varagona, 1985). Several primers pairs were developed based on differences between dominant Wx (Klösgen et al., 1986) and recessive wx published sequences (Fan et al., 2008). The alignment of these sequences showed only few differences between Wx and wx in the part of the gene considered (Fig 3.2). Amplicons of the same size for primers pairs W1, W2 and W3 (specific to the areas of exon 13, intron 13 and exon 14) were found in both waxy and non-waxy individuals; it illustrated the similarity in DNA sequences among homozygous Wx, heterozygous, and homozygous wx plants. This limitation may be related to deficits of the visualization method used as well since it is impossible to detect indels of one or only a few base pairs or single nucleotide polymorphism on agarose gels. Molecular marker P7P8 and S1S2 (Liu et al., 2007) were not usable on our material set and could not be implemented in a selection process for waxy here; this contradicts Liu’s conclusions presenting these primers pairs as universal markers for waxy/non-waxy selection. Indeed, in former study, P7P8 consistently amplified one DNA fragment in non-waxy material, while waxy genotypes exhibited a null-amplification; and S1S2 was the primers pair which amplified only waxy genotypes. In our study, only the primer pair W4 was identified as reliable on plant material.
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derived from two Chinese waxy*QPM hybrids CN35 and CN37 and could be routinely used to speed up selection for the waxy trait, by allowing an early screening at the seedling stage.

Since the waxy trait is due to the loss of function of a gene, several mutations may lead to this specific phenotype and may have been selected by Asian farmers over the last decades/centuries. The wide range of waxy mutations (Fan et al., 2008) complicates the development of one common universal marker for waxy, as it has been shown in this study. Applying known markers as MAS tool was not successful for waxy selection in the plant material of this study. One should consider other additional selection methods such as pollen staining or development of more accurate new primers pairs, specific to the material of interest. This would require additional efforts like evaluating different markers available in this region, sequencing the gene in different waxy and non-waxy plants and designing new specific markers. Another method could consist in following the technique implemented in rice with the use of single nucleotide polymorphism (SNP) instead of simple sequence repeats (SSR) markers for the discrimination of waxy and non-waxy genotypes (Gupta et al., 2001). Using SNP may be more effective since most of the waxy mutations identified so far were due to punctual mutations. However the high costs coupled to such a program may strongly limit its feasibility and availability for local Chinese and South East Asian breeding programs. However, the development of different markers, specific to the material of interest may still be possible and would substantially facilitate the breeding of waxy maize cultivars, be it conventional or by implementing doubled haploids.

3.5.3 Double recessive wx–o2 selection

The main objective of this project was to combine both recessive traits, waxy and opaque2, in order to enhance the nutritional quality of waxy maize. Screening the progenies separately for these two traits by the mean of molecular markers and pollen staining permitted to identify some doubled haploid plants, which carried both recessive alleles among the DH populations derived from five heterozygous Chinese waxy*QPM hybrids.

Plants of the three populations ETH3, ETH4 and ETH6 were weakened and partly damaged by the colchicine treatment (See chapter 2); only a small numbers of individuals remained at the end of the experiment. Thus, almost no double recessive wx–o2 plants were identified among plants derived from the three waxy*QPM hybrids CN3, CN4 and CN6. DH derived from the hybrid CN6 were particularly affected since no double recessive DH plant was
identified among them. The frequency of DH plants derived from CN3 and CN4 that carried both recessive alleles was low but appeared still acceptable.

The success of waxy selection in DH populations derived from CN35 and CN37 combined to the relatively good response to haploid induction and chromosome doubling of these heterozygous waxy*QPM hybrids (See chapters 1 and 2) produced an acceptable amount of double recessive DH plants. Five DH plants derived from the waxy*QPM hybrid CN37 and eight DH plants derived from CN35 were detected as double recessive \( wx-o2 \) (Table 3.2). These DH lines may be used for further development/breeding toward waxy maize of higher protein quality. The next step will be to investigate how these plants perform and to characterize their starch and amino acid profile to determine whether the goal of this project, namely developing waxy lines of higher protein quality for further breeding, has been met.
Chapter 4

Starch and protein composition in the endosperm of newly generated double recessive wx-o2 maize genotypes

* A publication based on this chapter has been submitted

Abstract

Granule bound starch synthase I (GBSSI) in plants is known as the main isoenzyme that catalyzes amylose synthesis. In waxy mutants (e.g. in wheat, maize, rice or potato) this isoenzyme is inactivated, which drastically reduces the synthesis of amylose and leads to starch exclusively consisting in amylopectin. A combination of waxy and quality protein maize (QPM) traits would improve the protein quality of waxy maize for human consumption. Double recessive waxy-QPM (wx-o2) genotypes were generated that showed indeed promising characteristics. The waxy trait was absolutely conserved and the amino acid profile was improved. But the vitreous kernel trait (due to additional modifier genes present in QPM) seemed to be lost in wx-o2 plant material. The content in specific amino acids like lysine, essential for human nutrition and usually deficient in current waxy maize germplasm, was substantially increased in double recessive wx-o2 genotypes compared to standard waxy maize. Amino acid profiles of all five double recessive wx-o2 genotypes were similar to milk. Therefore, they could truly contribute to meet human requirements in essential amino acids and thus reduce malnutrition.

4.1 Introduction

4.1.1 Maize starch components of common and waxy maize

In maize and many other plants, starch is stored in organs or tissues as the source of carbohydrates. The endosperm is the main compartment where starch is stored in maize kernels. Maize starch consists in two main components, amylose (AM) and amylopectin (AP). Amylose is a long unbranched polymer of glucose units linked by α-1,4 bonds. Amylopectin is a polysaccharide of highly branched polymers of glucose, where the glucose units are linked by α-1,4 bonds to form linear branches of 24 to 30 glucose units linked together by α-1,6 linkages. In common maize, starch consists in approximately 25% amylose and 75% amylopectin (James and Myers, 2009). In plants, the synthesis of amylose in amyoplasts is catalyzed by granule
bound starch synthases, especially by the granule bound starch synthase I (GBSSI). This
isoenzyme is encoded by the Wx gene (Klösgen et al., 1986) and highly activated in common
maize. In waxy maize, where the Wx gene is mutated, the isoenzyme GBSSI is inhibited which
leads to a very low AM content, usually less than 5% of the starch (Hylton et al., 1996; Liu et
al., 2007). The waxy mutation only affects the proportion of AM and AP in the starch of the
endosperm, pollen grains and embryo sac (Echt and Schwartz, 1981; Fergason, 2001). The
capacity of iodine binding and especially the difference in iodine affinity of AM and AP is
commonly used to estimate the starch composition and to differentiate waxy and non-waxy
maize genotypes. The affinity of AM is higher than those of AP (around 20.0g vs. 1.1g/100g),
which results in a black or dark blue color of the starch-iodine solution containing both, AM and
AP, compared to a brown color when only AP is present. To date, iodine staining is the main
method used to distinguish waxy maize in broad genetic backgrounds.

4.1.2 Amino acid profile in common and QPM maize

Maize kernels consist in two main compartments, the endosperm, accounting for 70 to
86% of the kernel weight, and the embryo, accounting for 7 to 22% of the kernel weight but the
contribution of these two compartments to the total amino acid content is different; the embryo
contains more essential amino acids on a percentage basis than the endosperm. Lysine and
tryptophan, known as the most essential amino acids in the view point of human nutrition
(Albanese et al., 1956; FAO, 1992; National Research Council, 1988), are present only in low
quantities in the endosperm of common maize (FAO, 1992). These two amino acid contents are
highly increased in specific maize varieties, so-called high quality protein maize varieties. The
maize quality protein mutant opaque2 (o2) was first pointed out 40 years ago (Mertz et al.,
1964) and led to the development of QPM maize. The o2 mutation nearly doubles the lysine
content of the maize endosperm. The extent of the increase in lysine of o2 mutants is highly
influenced by the genetic background (Moro et al., 1996). However, the o2 mutation also
negatively affects several agronomic traits including kernel characteristics and grain yield. The
lower yield of o2 genotypes was caused by an increased size of the endosperm which was soft,
chalky, dull and usually resulted in kernels that were more easily damageable, and thus more
susceptible to pests and/or fungal diseases (Bjarnason and Vasal, 1992; Vasal, S.K., 2001).
Various methods have been explored in order to overcome the shortcomings of o2 maize. The
germplasm developed by CIMMYT exhibits a hard endosperm in combination with the o2

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mutation; it was named quality protein maize (QPM), in opposition to the soft endosperm o2, called standard o2. In general, QPM maize contains about 55% more tryptophan, 30% more lysine and 38% less leucine than the wild type (Vasal, S.K., 2001). The vitreous endosperm of QPM caused by modifier gene(s) for o2 which is (are) not yet clearly identified, is the main difference between standard o2 maize and QPM.

Here the quality was evaluated of the plant material generated in this project with regard to starch composition and amino acid profile. We determined first the amylose/amylopectin content of the endosperm of waxy, QPM and double recessive wx-o2 plant material and second, the content in amino acids, especially in lysine and methionine, of selected double recessive wx-o2 genotypes. The interaction of the waxy and QPM traits on endosperm traits was also studied.

4.2 Materials and Methods

4.2.1 Plant material

Five double recessive wx-o2 genotypes were available: 1. Two doubled haploid (DH) double recessive lines, ETH37.1 and ETH37.2, derived from the heterozygous waxy*QPM hybrid CN37. 2. Two double recessive hybrids ETH35.1ETH37.1 and ETH37.3ETH37.2 (crosses between DH parental lines ETH35.1 and ETH37.1, and ETH37.3 and ETH37.2, respectively). 3. One double recessive backcross population ETH35.1ETH37.1*ETH37.1 (derived from DH parental lines ETH35.1 and ETH37.1). The parental double heterozygous hybrids CN35 and CN37, the waxy lines B601 and B602 as well as the QPM line B102 were used as standard controls for dominantly expressed standard kernel quality, waxy and QPM, respectively (all material was kindly provided by Dr. Wen Renlai, Guangxi Maize Research Institute, China). Moreover, two non-waxy genotypes, the Thai landrace LR59 and the common US-dent line Mo17 were controls for AM content.

4.2.2 Starch isolation for amylopectin and amylose quantification

4.2.2.1 Starch preparation

Starch was extracted according to Gutiérrez et al (2002). Five kernels of each genotype were bulked and steeped in 0.45% Na2S2O5 (at 50°C for 24h) before removing the embryo and the pericarp in order to keep only the endosperm. The steeped endosperms were ground with mortar and pestle. The wet powder was then blended with 10ml 0.05M NaCl for 1min. After filtration through a 50μm membrane, this material was purified five times with 5:1 (v/v) 0.05M
NaCl-toluene, by mixing with a vortex mixer for 15min and 5min centrifugation at 1000rpm. Purified samples were washed with 10ml distilled water once and once with 10ml acetone. The pellets were covered, left to dry at room temperature for 24h, powdered again and left to dry at room temperature for 72h.

### 4.2.2.2 Amylose – Amylopectin analysis

Amylose/amylopectin ratio was evaluated by spectrophotometry, according to Hoover et al (2001). The AM content was determined based on the formation of amylose – iodine complexes and their absorbance at a specific wavelength. Since starches were isolated with $\text{Na}_2\text{S}_2\text{O}_5$ and NaCl – toluene, the defatting step was omitted. Twenty mg (0.1mg precision) of each sample was mixed to 8ml of 90% dimethylsulfoxid (DMSO) (Sigma Aldrich, Buchs, Switzerland) and mixed vigorously for 2min. After 15min of intermittent mixing in an 85°C water bath, the tubes were cooled down to room temperature (around 45min). Thereafter the samples were diluted to 25ml with distilled water. One ml of the solution was further diluted with 40ml distilled water and 5ml iodine solution (2.5mM I$_2$/6.5mM KI), adjusted to 50ml with distilled water, and mixed vigorously. After 15min color development, the absorbance of each sample at the wavelength of 600nm was measured with a Hitachi U2000 spectrophotometer. Each sample was replicated nine times (except the non-waxy landrace LR59 and Mo17 (four replications), where the amount of starch available was limiting).

A standard curve was obtained on the mean of standard mixtures of commercial AM and AP (both purchased from Sigma Aldrich, Buchs, Switzerland) and provided the linear regression equation used to extrapolate the AM content of the samples. A series of 20mg mixtures of pure potato AM and pure maize AP at several relative proportions were prepared like the other starch samples for absorbance measurement. The standard curve was determined according to mean values (five replications) of the absorbance at 600nm of each standard AM/AP mix, ranging from 0 to 30% AM and from 70 to 100% AP. The standard curves for AM and AP (Fig 4.1) were based on the same data sources and complementary. Equations resulting from linear regressions determined the AM and AP contents as follows:

$$\text{OD} = 0.002.X_{\text{AM}} + 0.025$$  and  $$\text{OD} = -0.002.X_{\text{AP}} + 0.2261$$  \hspace{2cm} (r$^2$ = 0.998),

where OD (objective density) is the absorbance at 600nm and $X_{\text{AM}}$ and $X_{\text{AP}}$ are the AM and AP content (%), respectively.
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4.2.2.3 Starch granules observation

Isolated starch was immersed in 500 µl distilled water and 10 µl of this solution was stained by 20 µl I₂/KI (2.5mM I₂/6.5mM KI). The samples were observed under the 100x oil objective Olympus microscope.

4.2.3 Amino acid analysis

4.2.3.1 Flour preparation

Flour preparation was adapted from the method described by Wang et al. (2008). Five kernels of each genotype were pooled and soaked in distilled water for 2h in order to remove the pericarp and the embryo. Degermed endosperms were dried at 45°C until they reached constant weight (around 24h) and then milled by a metal ball into fine powder. The flour was stored at -20°C. Before each analysis, powder was defatted twice with 1ml hexane, centrifuged for 15min at 13000rpm and then dried at room temperature.

4.2.3.2 Amino acid analysis

Analysis of the amino acid profile was done by the protein analysis group of the Functional Genomics Centre Zurich. With the exception of tryptophan, all amino acids were...
analyzed by acid hydrolyzation (Huang et al., 2006). Defatted flour (10mg) was dissolved in 1ml deionized water and 10µl of each sample was hydrolyzed in 100µl 6N HCl buffer solution at 110°C for 24h. Twenty microliter of this solution was derivatized (50µl reaction solution including Norvaline as internal standard). High pressure liquid chromatography (HPLC) analysis was performed on 1ml of derivatized solution. Three different endosperm powder isolations were analyzed per genotype (except for ETH37.2 and B602, where duplicates of only one starch isolate were tested due to technical problems). In maize, lysine and tryptophan contents are highly correlated (Hernandez and Bates, 1969; Vivek et al., 2008), therefore an extra tryptophan analysis was not considered necessary for all genotypes.

4.2.4 Kernel hardness and opacity

Hardness of maize endosperm is visually illustrated by the opacity of kernels. Maize kernel phenotypes vary from hard (vitreous) to soft (opaque) endosperm (Pomeranz et al., 1984). Therefore, in this study, the kernel opacity was considered as an indicator of the hardness. A light table was used to evaluate the opacity of kernels selected according to the QPM selection protocol developed by CYMMIT (Vivek et al., 2008). This tool is commonly used to distinguish kernels with vitreous and opaque endosperm. As on the surface of the light table, only vitreous endosperms are translucent whereas the others are opaque, different types of endosperms, i.e. kernels, can be easily identified. Besides the molecular selection of homozygous o2 genotypes (conducted in the parental generation with molecular markers for o2, see chapter 3), QPM genotypes were selected according to the vitreousity, respectively the opacity, of the endosperm (Vivek et al., 2008).

4.2.5 Statistics

Analyses of the variance (ANOVA) of the AM, AP and amino acids contents were performed using the MIXED procedure of SAS® 9.1.3 (2004). The replicons were treated as random factor and genotypes were the fixed effects. Sources of variation and appropriate F ratios (Type III) were applied according to McIntosh (1983). Standard deviation, confidence interval and box plots were performed using the MEANS and BOXPLOT procedures of SAS® 9.1.3.

4.3 Results

4.3.1 Amylopectin and amylose content
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The AM and AP content of the endosperm of selected genotypes was determined by spectrophotometry according to the standard equations. In general, the absorbance of starch-iodine complexes was relatively low for all genotypes (Table 4.1). Two double recessive wx-o2 DH genotypes, ETH37.1 and ETH37.2, and the backcross population ETH35.1ETH37.1*ETH37.1 exhibited even slightly lower OD than the 100% commercial AP standard solution, which resulted in concentrations in AM below zero (-1.5 to -1.0%). This artifact may be related to the purity of the commercial AP used for the standard curve.

Table 4.1. Determination of amylose (AM) and amylopectin (AP) content (%) in endosperms of double recessive wx-o2 and control genotypes. The mean, standard deviation (SD) and confidence interval at 5% (CI) for the absorbance at 600 nm (objective density, OD) result from measurements of nine replicates per genotype.

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>OD</th>
<th>AM</th>
<th>AP</th>
</tr>
</thead>
<tbody>
<tr>
<td>ETH37.1 wxwxo2o2</td>
<td>0.023</td>
<td>-1.0</td>
<td>101.4</td>
</tr>
<tr>
<td>ETH37.2 wxwxo2o2</td>
<td>0.022</td>
<td>-1.5</td>
<td>101.9</td>
</tr>
<tr>
<td>ETH 35.1ETH 37.1 wxwxo2o2</td>
<td>0.028</td>
<td>1.5</td>
<td>99.0</td>
</tr>
<tr>
<td>ETH 37.3ETH 37.2 wxwxo2o2</td>
<td>0.026</td>
<td>0.5</td>
<td>101.6</td>
</tr>
<tr>
<td>(ETH 35.1 ETH 37.1) * ETH 37.1 wxwxo2o2</td>
<td>0.023</td>
<td>-1.0</td>
<td>101.7</td>
</tr>
<tr>
<td>CN35 WxwxO2o2</td>
<td>0.095</td>
<td>35.0</td>
<td>65.5</td>
</tr>
<tr>
<td>CN37 WxwxO2o2</td>
<td>0.093</td>
<td>34.0</td>
<td>66.3</td>
</tr>
<tr>
<td>B102 WxWxO2o2</td>
<td>0.110</td>
<td>42.5</td>
<td>57.2</td>
</tr>
<tr>
<td>B601 wwxO2O2</td>
<td>0.030</td>
<td>2.5</td>
<td>97.7</td>
</tr>
<tr>
<td>B602 wwxO2O2</td>
<td>0.032</td>
<td>3.5</td>
<td>96.9</td>
</tr>
<tr>
<td>Mo17 (Control) WxWxO2O2</td>
<td>0.100</td>
<td>37.0</td>
<td>64.1</td>
</tr>
<tr>
<td>LR59 (Control) WxWxO2O2</td>
<td>0.103</td>
<td>39.0</td>
<td>61.6</td>
</tr>
</tbody>
</table>

§, contents lower than 0% or higher than 100% may be related to the purity of the commercial AP used for the standard curve (See 4.4.1);
Means followed by the same letter within a column are not significantly different at the 0.05 probability level)

The two double recessive hybrids derived from DH lines, ETH35.1ETH37.1 and ETH37.3ETH37.2, showed slightly positive concentrations of 1.5% and 0.5% AM respectively. Accordingly, the AP content of these double recessive genotypes was almost 100%. The two standard waxy inbred lines, B601 and B602, contained more AM (2.5 and 3.5%, respectively), and therefore lower AP proportion (97.7% and 96.9% respectively) than double recessive genotypes. The QPM standard line B102 and the Thai landrace LR59 exhibited the highest AM
concentration (39% and 42.5%, respectively) combined to the lowest AP contents (57.2% and 61.6%, respectively). The two heterozygous Chinese waxy\textsuperscript{QPM} hybrids, CN35 and CN37, as well as the US dent maize Mo17, contained between 34.0 and 37.0% AM and between 64.1 and 66.3% AP.

Twelve genotypes and all standard samples were grouped according to their AM content (Table 4.1). The group of four selected double recessive \textit{wx-o2} maize genotypes (except ETH35.1ETH37.1) exhibited the lowest AM content, which was even lower than the standard solution of 0% AM. The two waxy maize inbred lines B601 and B602 grouped together with the other double recessive \textit{wx-o2} genotype ETH35.1ETH37.1 at a level corresponding to the standard samples 0 to 5% AM (Fig. 4.2). The AM contents of the heterozygous waxy\textsuperscript{QPM}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure4.2}
\caption{Objective density (OD) at 600nm of starch-iodine solutions of various maize genotypes reflecting their amylose content. Except Mo17 and LR59 (four replications), nine samples per genotype and five replications of each standard solution (AM/AP solutions ranging from 0/100\% to 30/70\% AM/AP) were measured. The box defines the first quartile (Q1) and the third quartile (Q3). The horizontal line inside the box presents the median. The lowest and highest value is connected to the box with the “whisker”.}
\end{figure}
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hybrids CN35 and CN37 and of Mo17 were similar, while the standard QPM B102 grouped with the other non-waxy maize genotype LR59. These two groups of non-waxy genotypes exhibited much higher AM contents (more than 30% of AM in the starch) than the waxy genotypes.

4.3.2 Endosperm opacity

To distinguish QPM from wild type maize, a light table was used for evaluating the hardness and opacity of the endosperms. Kernels of the standard QPM maize B102 (Fig 4.3, 2) transmitted the light more effectively than kernels of the heterozygous waxy*QPM hybrids CN35 and CN37 (Fig 4.3, 1 and 3). On the opposite, the endosperm of the waxy maize standard B601 (Fig 4.3, 4) was absolutely opaque. The kernels appearance of the five double recessive wx-o2 genotypes was alike those of waxy kernels; their endosperms were not translucent and these five double recessive genotypes showed dull opaque endosperms (Fig 4.3, 5 to 9).

![Fig 4.3. Maize kernels on light table.](image)

1: heterozygous hybrid CN35; 2: QPM standard B102; 3: heterozygous hybrid CN37; 4: waxy standard B601; 5 to 9: homozygous wxwxo2o2: 5. ETH37.1, 6. ETH37.2, 7. ETH35.1ETH37.1, 8. ETH37.3ETH37.2, 9. ETH35.1ETH37.1*ETH37.1
Table 4.2. Amino acid content (%) of the endosperm of selected maize genotypes (relative to the total amino acids content). Means and standard deviations result from analyses of three different starch isolations per genotype.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>ETH37.1</th>
<th>ETH37.2</th>
<th>ETH35.1</th>
<th>ETH37.1</th>
<th>ETH37.1</th>
<th>ETH35.1</th>
<th>ETH37.1</th>
<th>CN35</th>
<th>CN37</th>
<th>B102</th>
<th>B601</th>
<th>B602</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.77 ± 0.08</td>
<td>5.68±0.17</td>
<td>3.57 ± 0.07</td>
<td>4.45 ± 0.17</td>
<td>4.04 ± 0.03</td>
<td>2.94 ± 0.01</td>
<td>3.49 ± 1.00</td>
<td>4.80 ± 0.72</td>
<td>2.98 ± 0.44</td>
<td>2.89±0.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histidine</td>
<td>4.76 ± 0.11</td>
<td>5.53±0.18</td>
<td>5.25 ± 0.09</td>
<td>5.18 ± 0.35</td>
<td>5.16 ± 0.05</td>
<td>5.49 ± 0.05</td>
<td>5.47 ± 0.09</td>
<td>5.43 ± 0.12</td>
<td>5.50 ± 0.09</td>
<td>5.70±0.007</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serine</td>
<td>3.53 ± 0.25</td>
<td>4.73±0.01</td>
<td>3.74 ± 0.16</td>
<td>3.80 ± 0.54</td>
<td>3.44 ± 0.09</td>
<td>3.34 ± 0.30</td>
<td>2.84 ± 0.23</td>
<td>3.88 ± 0.82</td>
<td>3.01 ± 0.07</td>
<td>3.40±0.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arginine</td>
<td>3.75 ± 0.16</td>
<td>5.40±0.16</td>
<td>4.20 ± 0.21</td>
<td>5.19 ± 0.51</td>
<td>4.50 ± 0.21</td>
<td>3.25 ±0.11</td>
<td>3.23 ± 0.70</td>
<td>5.14 ± 0.42</td>
<td>3.61 ± 0.51</td>
<td>3.50±0.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td>13.84 ± 2.30</td>
<td>7.85±0.24</td>
<td>9.92 ± 0.44</td>
<td>10.45 ± 0.94</td>
<td>9.10 ± 0.55</td>
<td>6.70 ± 0.01</td>
<td>5.64 ± 1.43</td>
<td>6.72 ± 0.63</td>
<td>5.99 ± 0.46</td>
<td>7.09±0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>23.6 ± 0.84</td>
<td>18.84±0.58</td>
<td>21.04 ± 0.58</td>
<td>21.98 ± 1.23</td>
<td>23.3 ± 0.51</td>
<td>22.24 ± 0.86</td>
<td>20.5 ± 3.97</td>
<td>20.03 ± 1.76</td>
<td>20.7 ± 1.99</td>
<td>22.52±0.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Threonine</td>
<td>3.33 ± 0.05</td>
<td>4.60±0.14</td>
<td>3.80 ± 0.04</td>
<td>4.34 ± 0.25</td>
<td>3.84 ± 0.07</td>
<td>3.48 ± 0.00</td>
<td>3.4 ± 0.05</td>
<td>4.11 ± 0.28</td>
<td>3.45 ± 0.18</td>
<td>3.60±0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td>7.30 ± 0.80</td>
<td>6.84±0.19</td>
<td>7.03 ± 0.20</td>
<td>6.94 ± 0.52</td>
<td>7.37 ± 0.15</td>
<td>8.90 ± 0.18</td>
<td>8.61 ± 0.75</td>
<td>7.83 ± 0.63</td>
<td>8.94 ± 0.26</td>
<td>9.07±0.007</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proline</td>
<td>9.37 ± 0.35</td>
<td>12.78±0.47</td>
<td>11.17 ±0.15</td>
<td>11.27 ±0.46</td>
<td>11.94 ± 0.18</td>
<td>10.32 ± 0.04</td>
<td>11.56 ± 1.46</td>
<td>13.33 ± 0.31</td>
<td>10.96 ± 0.93</td>
<td>9.93±0.007</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lysine §</td>
<td>3.22 ±0.22</td>
<td>3.70±0.10</td>
<td>3.18 ± 0.09</td>
<td>4.00 ± 0.06</td>
<td>3.32 ± 0.25</td>
<td>1.93 ± 0.11</td>
<td>1.32 ± 0.25</td>
<td>2.92 ± 0.56</td>
<td>1.73 ± 0.12</td>
<td>2.16±0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tyrosine</td>
<td>2.00 ± 0.98</td>
<td>2.21±0.06</td>
<td>2.36 ± 0.06</td>
<td>1.44 ± 0.05</td>
<td>1.14 ± 0.06</td>
<td>2.94 ± 0.22</td>
<td>2.89 ± 0.22</td>
<td>2.26 ± 0.44</td>
<td>3.16 ± 0.30</td>
<td>2.80±0.007</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methionine §</td>
<td>0.77 ± 0.02</td>
<td>0.54±0.01</td>
<td>0.97±0.02</td>
<td>0.78±0.29</td>
<td>0.79±0.10</td>
<td>1.58±0.03</td>
<td>1.27±0.14</td>
<td>0.94±0.17</td>
<td>1.2±0.32</td>
<td>0.76±0.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Valine</td>
<td>4.98 ± 0.06</td>
<td>7.26±0.21</td>
<td>5.46 ± 0.26</td>
<td>5.49 ± 0.16</td>
<td>5.07 ± 0.12</td>
<td>4.85 ± 0.14</td>
<td>4.89 ± 0.14</td>
<td>5.45 ± 0.10</td>
<td>4.87 ± 0.19</td>
<td>5.02±0.007</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isoleucine</td>
<td>2.90 ± 0.17</td>
<td>3.05±0.08</td>
<td>3.18 ± 0.08</td>
<td>3.05 ± 0.39</td>
<td>3.04 ± 0.12</td>
<td>3.37 ± 0.11</td>
<td>3.44 ± 0.21</td>
<td>2.93 ± 0.25</td>
<td>3.41 ± 0.18</td>
<td>3.36±0.03</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leucine §</td>
<td>9.85 ±0.80</td>
<td>9.80±0.29</td>
<td>10.91 ±0.48</td>
<td>8.69±1.36</td>
<td>10.41±0.80</td>
<td>14.48±0.14</td>
<td>15.82±0.79</td>
<td>10.91±1.37</td>
<td>14.84±0.14</td>
<td>14.00±0.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>3.83 ± 0.31</td>
<td>3.32±0.10</td>
<td>4.15 ± 0.15</td>
<td>2.91 ± 0.79</td>
<td>3.51 ± 0.35</td>
<td>4.61±0.28</td>
<td>5.68 ± 1.73</td>
<td>3.25 ± 0.74</td>
<td>4.99 ± 0.98</td>
<td>4.31±0.02</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#, means and standard deviations result from analyses of two starch isolations.
§, means followed by the same letter within a line are not significantly different at the 0.05 probability level.
Fig 4.4. Starch granules at 100x magnification
A: standard QPM B102, with smaller granules and edges (arrows)
B to F: Double recessive wx-o2 genotypes, ETH37.1, ETH37.2, ETH35.1
ETH37.1; ETH37.3ETH37.2and
ETH35.1ETH37.1*ETH37.1, respectively
G: standard waxy B601
Besides exhibiting high AM content in its starch and vitreous endosperm, the starch granules of the QPM standard line B102 were altered (Fig 4.4, 2). B102 had smaller starch granules of irregular shape with edges meanwhile the standard waxy maize B601 (Fig 4.4, 4), and the five selected double recessive \(wx-o2\) maize genotypes (Fig 4.4, 5 to 9) had granules of bigger size, round shape and were especially characterized by a large amount of starch granules that had been broken during the extraction procedure. Broken granules with edges were found in waxy genotypes as well, which conferred them similar appearance to those of B102, i.e. the QPM standard genotype, but this may only be an artifact caused by the extraction procedure.

### 4.3.3 Amino acid profile of double recessive \(wx-o2\) maize

According to the results of amino acid analyses, the lysine proportion in the amino acid profile of homozygous recessive \(o2\) genotypes was modified (Table 4.2). Relative contents in other amino acids like methionine and leucine were changed too (Fig 4.5). Variations in lysine content depended substantially on the genetic background; the heterozygous waxy*QPM hybrid CN35 showed a significantly higher lysine content (1.93 \%) than CN37 (1.31\%), and the lysine content of the two Chinese waxy controls B601 and B602 differed strongly as well (Table 4.2).

For the selected double recessive \(wx-o2\) genotypes and the QPM standard B102, much higher proportions of lysine (between 2.9 and 4.0\% of the total amino acids) than for \(non-o2\) genotypes were found (Table 4.2). For the two double recessive DH lines derived from CN37, ETH37.1 and ETH37.2, the lysine content was 2.4 and 2.8 times higher than in the original heterozygous maternal donor hybrid CN37 (Table 4.3). No seeds of DH derived from CN35 were available for analysis since the DHs could not be multiplied by selfing due to asynchrony in male and female flowering times. The double recessive hybrid ETH35.1ETH37.1, which was obtained by crossing DH derived from CN35 and CN37, exhibited 2.4 times more lysine than the heterozygous hybrid CN37 and 1.6 times more than the hybrid CN35. Its lysine content was even 3.0\% higher than those of its paternal DH ETH37.1.

The highest lysine content found among the 12 genotypes was those of the hybrid between two double recessive DH lines derived from CN37, ETH37.3ETH37.2, where 4.0\% of the total amino acids of the endosperm consisted in lysine, which was 8.0\% higher than the lysine content of the best parent ETH37.2 and 3 times higher than the heterozygous hybrid of origin CN37 (Table 4.3). The backcross population ETH35.1ETH37.1*ETH37.1 showed a
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slightly higher amount of lysine than its parental genotypes (+4.2% compared to the maternal hybrid ETH35.1ETH37.1 and +3.0% compared to the paternal DH ETH37.1).

Table 4.3. Variation in lysine, methionine and leucine content (%) between five double recessive wx-o2 genotypes and their parental genotypes that were either heterozygous WxwxO2o2 (CN37 and CN35) or double recessive wx-o2 (ETH37.1, ETH37.2 and ETH35.1ETH37.1) genotypes.

<table>
<thead>
<tr>
<th>Comparison pair</th>
<th>Lysine</th>
<th>Methionine</th>
<th>Leucine</th>
</tr>
</thead>
<tbody>
<tr>
<td>ETH37.1/CN37</td>
<td>243.1</td>
<td>-39.0</td>
<td>-37.8</td>
</tr>
<tr>
<td>ETH37.2/CN37</td>
<td>280.0</td>
<td>-57.5</td>
<td>-38.0</td>
</tr>
<tr>
<td>ETH35.1ETH37.1/CN37</td>
<td>240.5</td>
<td>-23.5</td>
<td>-31.0</td>
</tr>
<tr>
<td>ETH35.1ETH37.1/CN35</td>
<td>164.7</td>
<td>-38.6</td>
<td>-25.7</td>
</tr>
<tr>
<td>ETH35.1ETH37.1*ETH37.1/ETH37.1</td>
<td>103.1</td>
<td>101.8</td>
<td>-34.2</td>
</tr>
<tr>
<td>ETH35.1ETH37.1*ETH37.1/ETH35.1ETH37.1</td>
<td>104.2</td>
<td>-18.7</td>
<td>-4.6</td>
</tr>
<tr>
<td>ETH37.3ETH37.2/ETH37.2</td>
<td>108.0</td>
<td>144.1</td>
<td>-11.3</td>
</tr>
<tr>
<td>ETH37.3ETH37.2/CN37</td>
<td>302.0</td>
<td>-38.6</td>
<td>-45.0</td>
</tr>
</tbody>
</table>

Fig 4.5. Proportion (%)(w/w) of some amino acids, threonine (Thre), alanine (Ala), lysine (Lys), methionine (Met), valine (Val), isoleucine (Ileu) and leucine (Leu) in different genetic backgrounds (relative to the total amino acid content). Three replicates per genotype were considered, except for ETH37.2 and B602 (two replicates).

Methionine is another important amino acid for human nutrition. A trend to decrease this amino acid in recessive o2 genotypes was detected (Table 4.3). Compared to the heterozygous hybrid CN37, the two selected double recessive DH, ETH37.1 and ETH37.2, exhibited methionine contents sharply lowered by 39.0% to 57.5%, respectively. The methionine content
of the double recessive hybrid ETH35.1ETH37.1 was lower than those of the waxy*QPM hybrids of origin CN35 and CN37 (38.6 and 23.5%, respectively). In comparison with the double recessive backcross population ETH35.1ETH37.1*ETH37.1, the methionine level in this maternal hybrid ETH35.1ETH37.1 was a bit higher (Table 4.3). On the opposite, the hybrid ETH37.3ETH37.2 had higher methionine content (+44.1%) than its paternal DH line ETH37.2.

Leucine content was substantially reduced in the double recessive wx-o2 genotypes (Table 4.2). Compared to corresponding heterozygous waxy*QPM hybrids, leucine content of the double recessive hybrid ETH37.3ETH37.2 decreased by 45% and those of the two DH lines ETH37.1 and ETH37.2 and of the double recessive ETH35.1ETH37.1 from 31.0 to 38.0% (Table 4.3). Compared to the recurrent parent ETH37.1, the backcross population ETH35.1ETH37.1*ETH37.1 had 34.2% less leucine; however this decrease represented only a decrease of 4.6% when compared to the maternal double recessive hybrid ETH35.1ETH37.1. Nevertheless, leucine is not a limiting amino acid in maize and its extent in these genotypes was still sufficient with regard to human nutritional needs.

4.4 Discussion

4.4.1 Starch components in double recessive wx-o2 genotypes

Waxy maize is known as the pure source of AP for the industry. The standard Chinese waxy maize germplasm contained around 97% of AP, meanwhile the double recessive wx-o2 genotypes obtained in the frame of this project had up to 100% of AP. The AP content of starch of selected double recessive wx-o2 maize genotypes was sometimes even higher than those of the commercial standard of 100% AP according to the absorbance of starch-iodine complexes at 600nm. Since the standards were purchased (waxy maize AP, Fluka-Sigma Aldrich, Buchs, Switzerland), this observation may be related to two different reasons, first to different starch isolation processes and second to the fact that commercial AP originates from waxy maize, which was reported not to consist in 100% AP, like it is assumed in theory, but may contain up to 5% AM (Nelson and Pan, 1995; Nelson, O.E. and Rines, 1962). This was confirmed in our study as well, where up to 3.5% AM was found in standard Chinese waxy maize. The quality of commercial AP standards may explain why the AP content of some double recessive wx-o2 maize genotypes appeared to be 1 to 2% higher than the 100% AP standard.
The QPM line B102 exhibited the highest AM content (42.5%), which is unusual for maize, except for high amylose mutants, like *amylose-extender (ae)* maize, where amylose can contribute to up to 80% of the starch (Fergason, 2001). Gibbon et al. (2003) showed that, in some QPM genotypes, the level of GBSSI was increased, which may explain the higher AM content. Moreover, the starch structure of some QPM was changed and exhibited shorter AP branches and increased starch granule swelling compared to their *o2* counterparts or to wild types. This may eventually lead to AM-like AP, which may be the reason why the AM content measured in QPM was much higher than the theory predicted and thus higher than it may really be. However, none of the selected double recessive *wx-o2* genotypes showed abnormally high AM content, which could be interpreted as AM-like AP. According to light microscopy, it was obvious that the starch granules of the QPM standard genotype B102 were smaller, with edges and more resistant (less granules altered by the starch extraction procedure) than those of double recessive *wx-o2* genotypes, which were bigger, round and often broken.

Combining *o2* with other recessive genes has been done since the *o2* mutation was discovered; in *sugary high quality protein* maize, combining the *sugary2 (su2)* and the *o2* mutation, slightly translucent, dull endosperms, i.e. less opaque kernels, occurred. Especially, the lysine content of these double mutants was slightly higher than those of standard *o2* but it was coupled to a decrease in yield of around 20% (Garwood and Creech, 1972; Glover et al., 1975; Glover and Tosello, 1973; Thomas et al., 1976; Vasal, S.K., 2001).

In waxy maize, the function of GBSSI is deactivated (Echt and Schwartz, 1981; Klösgen et al., 1986; Nelson, O.E. and Rines, 1962), and amylose is not synthesized, while in QPM, the content of GBSSI in soluble non-zein protein fraction is increased (Gibbon et al., 2003). These two contradictory effects of *o2* and *wx* illustrate the possibility of antagonistic expression of the mutated genes and the fact that only one of the two traits of interest may be activated in the combination of waxy and QPM. The results of double recessive *wx-o2* genotypes confirmed on the one hand the pure waxy appearance (non-altered starch structure with high AP content) and on the other hand the opacity of *wx-o2* kernels, which was clear evidence for GBSSI deactivation.

Although the hard kernel trait of QPM due to modifier gene(s) of *o2* seemed to be missing in double recessive *wx-o2* genotypes, the combination of two recessive genes in one genotype showed the possibility of improving the quality of waxy maize in both, nutritional
(high lysine content) and physical (pure AP starch) traits. Furthermore, since the main aim of this project was to develop a better food resource, soft kernels of waxy maize might be more acceptable for human consumption than harder kernels of QPM. Nevertheless, agronomic and organoleptic quality of the plant material developed in this project was not evaluated and would probably require additional breeding efforts to meet requirements of both, growers and consumers.

4.4.2 Variations in essential amino acids of double recessive wx-o2 genotypes

With regard to nutritional value, lysine and tryptophan are the most lacking essential amino acids in maize, except in standard o2 maize and QPM. The aim of this project was to improve the protein quality in waxy maize by introgressing the o2 trait. The maize endosperm content in lysine and in other amino acids varied strongly among genotypes depending on the genetic background, which corroborated previous findings by Moro et al (1996). In homozygous recessive o2 genotypes, not only the lysine content was increased, the content in other amino acids, such as threonine, alanine, methionine or leucine, varied accordingly as well (Fig 4.5). Compared to normal maize, the whole amino acid profile was modified in double recessive wx-o2 genotypes. The most obvious increase was observed for lysine (increase in tryptophan is assumed as well since these two amino acids contents are positively correlated in maize) compensated by sharp reduction in leucine and methionine. Slight modifications in threonine and valine were observed as well. In QPM and in o2 maize, zein protein fraction is reduced and the fractions of proteins belonging to non-zein groups are increased (Moro et al., 1996). Non-zein protein groups are known to be much richer in amino acids like lysine or tryptophan than zeins (Shukla and Cheryan, 2001). The enhancement/reduction in amino acid contents clearly illustrates the variations in relative fractions of zein and non-zein proteins in double recessive wx-o2 genotypes. These results were consistent with others studies, where variations in zein/non-zein protein fractions were reported in various high quality protein maize (in o2 maize and in maize with other quality protein mutations) as well (Geetha et al., 1991; Geraghty et al., 1981; Hunter et al., 2002).

Among the five selected double recessive wx-o2 genotypes, the DH ETH37.2 and its hybrid ETH37.3ETH37.2, showed the highest increase in lysine, valine and threonine content compared to other double recessive genotypes and to the QPM standard B102. As a
consequence, the proportions of other amino acids like methionine, alanine and leucine decreased more in these genotypes than in other \(wx-o2\) genotypes. One can hypothesize that in ETH37.2, and thus, partly in ETH37.3ETH37.2, where half of the genome comes from ETH37.2, other loci (or genetic factors), which could interact with \(o2\) and strengthen the expression of the \(o2\) trait, may be activated.

According to Scott et al. (2004), the level of tryptophan in QPM is negatively correlated with the endosperm vitreousity, which means that kernels with lower tryptophan content are usually more vitreous on the light table than wild type maize. This phenomenon was not observed on recessive \(o2\) and \(wx\) material obtained in this study, where some double recessive \(wx-o2\) genotypes were opaque as well on the light table even they have lower lysine content than the others. However, since the tryptophan content was not directly evaluated here, it was impossible to determine whether it was really increased (as positively correlated to lysine content). An impact on the tryptophan content may be as well related to the loss of typical QPM traits in double recessive \(wx-o2\) combinations reported in previous paragraph. In QPM, modifier gene(s) act(s) in complementation of the \(o2\) gene to countermand pleiotropic effects of \(o2\). Since this study focused only on \(o2\) and not on the modifier(s), they may probably be partly missing or inactive in the plant material obtained in this project.

According to the World Health Organism (WHO), the requirement in lysine for adults can be met by consuming milk, meat and egg on a regular basis. When used as staple food, cereals like maize, rice and wheat are deficient in lysine and cannot fulfill the needs in this amino acid (WHO, 2007a). This is especially the case for children and infants whose demand in lysine and other essential amino acids is proportionally much higher than those of adults (Table 4.4).
Therefore, the amino acid supply of children’s diet should be proportionally higher than what is recommended for adults. In normal maize, except for tryptophan and lysine, the amino acid contents are higher than required, which makes it a relatively balanced staple food if the

### Table 4.4. Human requirements (mg/kg/day) in different amino acids (Source: WHO, 2007a, b) and amino acid supply of milk, double recessive wx-o2 and standard waxy maize. The values for double recessive maize represent the range (min-max) extrapolated from five wx-o2 genotypes.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Supply (mg/100g)</th>
<th>Requirements (mg/kg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Adults</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&lt;1yr</td>
</tr>
<tr>
<td>Histidine</td>
<td>Milk</td>
<td>118</td>
</tr>
<tr>
<td></td>
<td>wx-o2 maize</td>
<td>300-1000</td>
</tr>
<tr>
<td></td>
<td>wx maize B601</td>
<td>240</td>
</tr>
<tr>
<td></td>
<td>Milk</td>
<td>200-500</td>
</tr>
<tr>
<td></td>
<td>wx-o2 maize</td>
<td>430</td>
</tr>
<tr>
<td></td>
<td>wx maize B601</td>
<td>540-1600</td>
</tr>
<tr>
<td></td>
<td>Milk</td>
<td>248</td>
</tr>
<tr>
<td>Leucine</td>
<td>wx-o2 maize</td>
<td>300-630</td>
</tr>
<tr>
<td></td>
<td>wx maize B601</td>
<td>300-800</td>
</tr>
<tr>
<td></td>
<td>Milk</td>
<td>153</td>
</tr>
<tr>
<td>Lysine</td>
<td>wx-o2 maize</td>
<td>400-1200</td>
</tr>
<tr>
<td></td>
<td>wx maize B601</td>
<td>400-1200</td>
</tr>
<tr>
<td></td>
<td>Milk</td>
<td>255</td>
</tr>
<tr>
<td>Threonine</td>
<td>wx-o2 maize</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>wx maize B601</td>
<td>NA</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>wx-o2 maize</td>
<td>340-1200</td>
</tr>
<tr>
<td></td>
<td>wx maize B601</td>
<td>410</td>
</tr>
</tbody>
</table>

NA, not available

\( ^{s} \) tryptophan values for wx and wx-o2 maize were estimated as one third of lysine supply (Scott et al., 2004; Vivek et al., 2008)
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supply in lysine and tryptophan is ensured by the consumption of other nutrient sources like milk or meat (WHO, 2007a).

In double recessive \(wx-o2\) genotypes, the lysine content is quite high, around 300mg/100g of endosperm flour for most of the genotypes with the exception of ETH37.2 where it was even the double (600mg/100g) (data not showed). Furthermore, not only the endosperm of maize is consumed but the whole kernel, which means that the embryo, characterized by a balanced amino acid profile, represents as well a substantial part of the diet and contributes to higher supply in essential amino acids. Compared to human needs in amino acids, double recessive \(wx-o2\) plant material developed in the frame of this project seem able to cover humans’ requirements, especially for children and infants. Its nutritional value with regard to essential amino acids is similar to those of pasteurized cow milk.

4.5 Conclusion

The aim of the entire project was to develop waxy maize with improved protein quality, i.e. improved amino acid profile with regard to human nutritional requirements, by QPM introgression using the method of doubled haploids production. The \(wx-o2\) genotypes developed contained both, high amylpectin, related to the waxy trait, and high lysine, related to the \(o2\) trait, which illustrates the possibility to combine both traits with this technique. The amino acid profile of these genotypes was substantially improved and could be of major interest for regions, where waxy maize is consumed as staple food and where other protein sources like milk or meat are limiting, e.g. in marginal areas of South East Asia. Although high protein quality has been achieved in double recessive \(wx-o2\) genotypes, the expression of \(o2\) and the control of modifier gene(s) for \(o2\) in these combinations seem complicated and would require additional breeding toward varieties of satisfying agronomic and organoleptic traits. Nevertheless, the achievements of this project are promising and encourage going farther by producing and evaluating other double recessive DH derived from more diverse waxy and QPM sources to develop new waxy varieties of higher nutritional value.
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General conclusion

5.1 Global malnutrition situation

In 2009, the Food and Agriculture Organization of the United Nations (FAO) estimated that, worldwide, about 1.02 billion people, i.e. one sixth of world’s population, were undernourished (FAO, 2009). This is the highest number for the entire human history. It has been demonstrated that children who were undernourished in their first five years of life, are more often affected by illness, and exhibit usually a lower cleverness and shorter mean life duration. Moreover, malnutrition can even affect the next generation (UNICEF, 2009), which is even more alarming for developing countries, where the majority of the undernourished children under five years old are living.

5.2 Quality protein maize and waxy maize

5.2.1 Quality protein maize

Breeders succeeded in improving maize with respect to the content in the two most essential amino acids for human nutrition, namely lysine and tryptophan, which are usually present in insufficient quantities in regular maize. This maize, first called opaque2 maize, was then bred toward better agronomic traits and kernel vitreousity. The introgression of modifier genes led eventually to the so-called quality protein maize (QPM) of higher protein quality and satisfying agronomic traits. QPM is more acceptable for farmers as its higher yielding and more resistant to pests and diseases than the standard opaque2 maize developed first. Since the number of people suffering from malnutrition is constantly increasing, the cultivation of QPM in developing countries should be promoted.

5.2.2 Waxy maize

Waxy maize is famous because of its particular starch, consisting in pure amylopectin, which confers its special physical and rheological properties and is responsible for its massive use in food, paper, textile and adhesives industries (Kennedy and Fischer, 1984; Kirby, 1986; Moore et al., 1984). This specialty maize came from Asia, where waxy maize is still used as a vegetable food, especially for some groups of populations, for whom it is the main diet component. There, conventional breeding with backcrossing and recurrent selection is still used as the main breeding method. New goals combined to new methods in breeding waxy maize
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with improved protein profile and enhanced nutritional quality would help to substantially prevent malnutrition in Asia and also in many other areas worldwide.

5.3 Improvement of the protein quality in waxy maize

The improvement of protein quality of waxy varieties was not seriously studied until 2005, when several groups in Thailand, Vietnam, China and Switzerland started research programs. Different methods to reach the goal of creating high quality protein waxy maize are currently explored. New double recessive wx-o2 maize lines were developed with the combined means of conventional breeding and marker assisted selection. However, none of these lines are clearly recorded or published yet, thus no further detailed information is available concerning this material.

5.3.1 Doubled haploid (DH) production

The technique of producing doubled haploids (DH) was rapidly adopted in maize breeding after in vivo haploid induction was successfully introduced. Although the mechanism of haploid induction with so-called inducer lines has been intensively studied, the underlying physiological principles still remain unclear. It is assumed that one of the two sperm cells of the inducer lines is deficient and thus not able to fuse with the egg cell, while the other cell is still able to fuse with the central cell and form the triploid endosperm.

5.3.1.1 Production of haploids from tropical and subtropical waxy*QPM hybrids by in vivo gynogenesis

In in vivo gynogenesis, haploid seeds are produced by pollination with special maize lines called inducer lines. So far, the modern European inducer line RWS showed the best haploid induction rate with an average of 8% haploid seeds among the progeny and a low rate of misclassification according to anthocyanin pigmentation when used on European dent maize. With flint plant material, numerous false positive cases appeared when selecting haploids just visually (Röber et al., 2005). Until now no study was published on the efficiency of RWS on tropical and subtropical plant material. Only in vivo androgenesis with the haploid inducer line W23 was investigated on tropical maize and resulted in very low haploid induction rates (0.94% in average) (Belicuas et al., 2007). Another disadvantage of the system mentioned in this former study was the genetic dependent expression of anthocyanin pigmentation, which complicated the visual selection of haploid seeds after induction.
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In our project, the heterozygous waxy*QPM hybrids were induced not just by one inducer line, RWS, but also by two other modern European inducer lines, RWK76 and RWK76RWS, expected to be more efficient (Geiger, 2009). As reported before, see above, on tropical maize induced with the inducer line W23, the expression of anthocyanin pigmentation in the progenies resulting from crosses between the three inducer lines and subtropical/tropical waxy*QPM hybrids varied strongly here, too. Since not only the special “red crown” and “red embryo” traits (due to the $R1\cdot nj$ gene of the inducer lines) were affected, but also the expression of the $Pl1$ (purple plant) gene (the additional gene for anthocyanin pigmentation) was influenced as well, the haploid selection procedure at first took longer than expected. The occurrence of the purple stem trait in waxy plant materials, and therefore in waxy*QPM hybrids too, complicated sensibly the selection process of haploid seeds among induced seeds. About half of the seeds derived from these plant materials after the induction with inducer lines RWS, RWK76 and RWK76RWS carried unpigmented embryo and unpigmented endosperm. According to Geiger (2009), these unpigmented seeds were considered as “out-crossed or selfed” seeds, when they occurred in European flint-dent haploid induction procedures. However, under controlled growing conditions in greenhouse and relying on the fact that the waxy*QPM hybrids were detasseled before pollen shedding, the occurrence of seeds resulting from “out-crosses” or “selfings” was impossible. In our study, the presence of unpigmented seeds among the progenies is more likely due to the impact of maternal donor genetic, which may interfere or inhibit the expression of the anthocyanin pigmentation. Similar cases were as well mentioned in 50 tropical maize races originating from Latin America that were induced by the same inducer line(s) (Blanco and Smelser, 2009, unpublished data). Nevertheless, in our study, the control of the seeds by flow cytometry permitted eventually to get precise data on their ploidy level and to select haploid seeds among the progeny. In the study by Blanco and Smelser, haploid seedlings were only selected by visible “seedling vigor”, which was not as reliable as flow cytometry (FCM) and may not permit to distinguish all cases of misclassification.

Although the $R1\cdot nj$ gene was not always reliable here, it still plays an important role in the initial selection of putative haploid seeds, because all seeds with red embryos were discarded since they were surely diploid. Therefore, with plant material, which is highly susceptible to carry the anthocyanin inhibitor allele $C1^I$, the initial step would be to eliminate diploid seeds
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(with pigmented embryo) and then to control the ploidy level of putative haploid seeds (unpigmented embryo) by FCM.

Gynogenesis on tropical and subtropical waxy*QPM maize with modern European inducer lines led to higher and more acceptable haploid induction rates (ranging from 9.6 to 13.3%) than androgenesis with the inducer line W23. This result proved the high efficiency of these inducer lines for producing haploids even when they were derived from various genetic backgrounds. Haploid induction rates observed on subtropical and tropical maternal donor hybrids depended strongly on both, the environment and the genetic of the donor. Hence, for higher efficiency of in vivo gynogenesis methods with specific subtropical/tropical materials the impact of the donor background should be well considered. Subtropical/tropical maize remains a very important source of diversity for modern maize breeding and developing new improved inducer lines or new haploid identification systems that would better suit to subtropical/tropical maize may be of high interest not only for local breeders.

5.3.1.2  Chromosome doubling on subtropical/tropical plant material

As modern inducer lines had not been used before to create haploids with tropical and subtropical maize, especially with waxy and/or QPM, the technique of in vivo chromosome doubling on this material was quite uncertain, too. Colchicine is still the most common chemical used in scientific institutions for maize chromosome doubling, both under in vitro and in vivo conditions. We focused on haploid seeds derived from Chinese waxy*QPM hybrids for the experiment on chromosome doubling because they exhibited the highest haploid induction rate. After having been treated with colchicine (0.06%) for 12h (Deimling et al., 1997), these seedling populations survived quite well and showed an acceptable rate of chromosome doubling according to results of flow cytometry. Although the chromosome set of haploid plants was doubled, their fertility remained low since the long anthesis silking interval (ASI) observed usually in waxy*QPM hybrids was coupled to very low quantities of pollen produced by DH plants; only a few DH plants could eventually be selfed and produce seeds. In studies of the germplasm enhancement of maize project (G.E.M.Project), similar treatment for chromosome doubling of tropical haploid plants was considered (Blanco and Smelser, 2009 unpublished data). There, doubling rates of around 19%, evaluated on the number of successful selfing events (not assessed by FCM), were achieved.
The problem of long ASI of subtropical/tropical material might be overcome by cultivating treated plants in more optimal growing conditions. Nevertheless, with the fertility rate (rate of plants that produced seeds) of haploid plants from 13.6% to 33.0% achieved in our project, the application of in vivo gynogenesis to tropical and subtropical waxy*QPM breeding exhibits promising potential for the future.

5.3.2 Selection of waxy and quality protein seeds

The maize waxy gene is located close to the centromere of chromosome 9 and its \( wx \) mutant is one of the most difficult to identify (McClintock, 1952). Many different mutants of waxy maize were identified over the years (Nelson, O. E., 1968; Nelson, O.E. and Rines, 1962). The Chinese maize waxy mutant is assumed to result from the deletion of several nucleotides in the wild-type waxy gene of common cultivated maize (Fan et al., 2008). Since the mutation appears to be very punctual, the development of specific markers for the marker assisted selection of waxy maize was complicated. We could not develop any universal molecular marker able to discriminate all the waxy from normal maize genotypes. Only the recessive waxy mutation of two haploid populations ETH35 and ETH37 could be selected by the mean of marker W4. This marker was not discriminating waxy and non-waxy in the other genetic backgrounds investigated in this study. Our result once again strengthened the importance of pollen (or starch) staining as the most efficient way to select waxy maize genotypes with a broad genetic background.

5.4. Quality of protein and starch in double recessive waxy*QPM genotypes

The combination of waxy and QPM seems ideal for improving the food quality and security in many developing countries. Nevertheless, the possible interaction between the waxy and the QPM traits is not yet well understood and remains an interesting field of work since the activities of granule bound starch synthase I (GBSSI) in waxy and QPM appear contradictory. In plants GBSSI is known as the main isoenzyme catalyzing the synthesis of amylose (Nelson and Pan, 1995; Nelson, O.E. and Rines, 1962). In waxy mutants (not only in maize but also in wheat, rice or potato), almost no GBSSI is detected while proteomic analysis revealed that GBSSI activity was increased in QPM compared to the wild-type.
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Here, we did not focus on measuring the content or activity of GBSSI in waxy*QPM plants (double recessive \textit{wx-o2} genotypes), but we considered amylose and amylopectin content as well as kernel opacity. Among the selected double recessive \textit{wx-o2} genotypes, amylose was almost not detected, which may indicate inactivity of GBSSI in these genotypes. Thus, the higher level of GBSSI related to the QPM trait seemed to be missing in double recessive genotypes derived from waxy*QPM hybrids. On the other hand, the observation of kernels on the light table revealed that none of the selected double recessive \textit{wx-o2} genotype possessed a vitreous endosperm, which means that the vitreous kernel trait related to QPM was not expressed either. This can be caused by the waxy trait, as waxy maize kernels are described as dull, opaque and with pure amylopectin starch, or to the absence or inactivity of additional modifier gene(s) carried by QPM.

Nevertheless, the main objective of this project was not to reproduce QPM maize with all its characteristics but to produce waxy maize with higher quality of protein and this was successfully achieved as confirmed by qualitative analyses of selected double recessive \textit{wx-o2} genotypes. The two most important amino acids, lysine and tryptophan, were substantially increased (almost doubled) in double recessive \textit{wx-o2} maize compared to standard waxy maize and even slightly higher than for standard QPM.

Furthermore, researchers at Guangxi Maize Research Institute, China (Zhang et al., 2008) found some waxy maize varieties that exhibited \textit{per se} already high content in essential amino acids, comparable to those of QPM. Using this kind of material as the waxy maternal donor for further development of double recessive DH \textit{wx-o2} plant material may improve even more its protein quality of the product.

5.5 Summing it up

This was the first application of \textit{in vivo} gynogenesis toward development of subtropical/tropical double recessive \textit{wx-o2} genotypes. The results illustrated the high potential of practical application of this method. However, transferring this technique to local breeding programs may take longer time since modern inducer lines are mostly temperate plant material and may not be well-adapted to subtropical/tropical conditions. Therefore, a new program that aimed to develop new inducer lines for tropical conditions and tropical donor material (with other visual markers for haploid selection) is currently carried out at CYMMIT in cooperation between CYMMIT and the University of Hohenheim.
Although high haploid induction rates were achieved, the bottleneck remains chromosome doubling with colchicine, which substantially impacts the regeneration rate of doubled haploid plants. Hence, the identification or development of alternative chemical(s) for chromosome doubling in tropical and subtropical maize should be one of the priorities in order to improve the method and allow the routine implementation of in vivo gynogenesis in exotic maize breeding as well. Other chemicals, more efficient in chromosome doubling, less toxic and cheaper, were identified/developed and are currently widely used by breeding companies. However, these products remain confidential and could not be tested in the frame of this PhD project. We were not able to test different chemicals or to extend our work on more subtropical and tropical plant material in order to get more representative data. This information still remains interesting since chromosome doubling rates seem easily improvable, especially on tropical and subtropical material, by the implementation of these alternative chemicals in the future.

As maize in general and waxy maize in particular is already and will always be one of the most important sources of carbohydrates for human nutrition, hence improving maize contents in the two limiting amino acids lysine and tryptophan could ensure better nutrient uptakes for millions of poor people in many developing countries and substantially decrease malnutrition all over the world. Even though the technique of producing doubled haploids derived from tropical and subtropical waxy*QPM hybrids with modern inducer lines is not yet optimal, the haploid induction rates observed on the waxy*QPM hybrids CN35 and CN37 were quite remarkable and promising. Double recessive wx-o2 genotypes derived from waxy*QPM hybrids showed that the waxy trait was expressed on the endosperm and that the protein quality related to o2 was clearly improved though the modified kernel traits derived from QPM were partly lost. Finally, this project illustrated the possible implementation of these techniques for rapidly producing better food resources for human consumption.
References


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Acknowledgement

I would like to express my sincerest thanks to Prof. Peter Stamp for giving me the opportunities to work in this topic in his group. Peter, thank you so much for your invaluable advices, your understanding and your big hugs throughout my study. My study would never be ended without your endless support and it would be so boring life in the office and greenhouse without all of your jokes in the stress PhD study.

I very much thanks to Dr. Magali Munsch for her advices, discussions and especially for the editorial support. Mag, I really appreciate your help and all the time you spent in my diss.

I would like also to thank Dr. Ingrid Aulinger for her guidance at the beginning of my PhD. My big thanks to my boss, Dr. Le Huy Ham for first introducing me to Peter and also accepted to be my co-examiner. I am grateful to my co-examiner Dr. Karl-Heinz camp for spending his time to read my dissertation. Many many thanks to Susanne Hochman, Marianne Wettstein-Bättig, Brigitta Herzog for helping me with the tedious laboratory and greenhouse work. My study will take much much longer if I do not have the help from you. P’Kung and Lek, thanks so much for sharing with me the happiness and also my sadness time. I would never forget your caring to me. Susanne, Debby, Niclas, David, Dominique, Rainer, Chritophe, Regina, Andi, Jörg, Juan, Chaminda… I enjoyed our chatting, discussion and it will be unforgettable memories for me and hope will have more time to work with you

My big thanks to the excellent administration works by Susanne Stamp, Inge Demetrious and Madeleine Neuhausen.

It would be much more difficult without the encouragement from my family, my family-in-law. Con cảm ơn bố mẹ và cả nhà đã luôn yêu thương, ủng hộ và giúp đỡ con mọi lúc, mọi nơi. My great thanks to my hubby. It was really hard time for us to stay separately in two different countries and I will always remember our weekend chat in the lab and the night trains connect Zurich to Vienna. Anh yêu, cảm ơn tình yêu, sự thông cảm và sự giúp đỡ không ngừng của anh trong cuộc sống cũng như trong công việc. Cảm ơn anh dà luôn ở bên cạnh em những lúc khó khăn nhất, giúp đỡ em có thêm động lực để có được như hôm nay. Thank you also all of my Vietnamese friends around

Last but not least, with a deep sense of appreciation and gratitude I thank to Prof Geiger, Dr. Wen, Dr. Jampatong and Dr. Le for providing the materials and to the Swiss Federal Commission of Scholarship for Foreign Student (FCS) did support my finance for the first two years.
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