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Genetic marker analysis in potato for extreme resistance (*Ry_{sto}*) to PVY and for chip quality after long term storage at 4°C

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Contents

Abbreviations

acl	*Solanum acaule*
adg	*Solanum tuberosum* ssp. *andigena*
dms	*Solanum demissum*
BSA	Bovin serum albumin
cDNA	Complementary DNA
cM	centi Morgan
CTAB	Cetyltrimethylammoniumbromid
dms	*Solanum demissum*
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylenediaminetetraacetic
F_1	Filial generation 1
M	Molarity
MQM	Multiple QTL Model
NADP	Nicotinamide Adenine Dinucleotide Phosphate
r	Correlation coefficient
SCAR	Sequence Characterized Amplified Region
SDS	Sodiumdodecylsulfate
SSC	Saline-Sodium citrate
STM	Solanum Tuberosum Microsatellite
sto	*Solanum stoloniferum*
TE	Tris-EDTA
TBE	Tris Boric acid EDTA
tbr	*Solanum tuberosum*
Tris	Tris-(hydroxymethly)-aminomethan
U	Unit
vrn	*Solanum verneii*

1 Introduction

1.1 Taxonomy and importance of the potato crop

The potato originated in the Bolivian–Peruvian Andes and belongs to the *Solanaceae* family and the *Solanum* genus containing about 1000 species. The subgenus *Petota* is divided into two subsections *Estolonifera* (non-tuber bearing) and *Potatoe* (tuber bearing). In addition to six other cultivated species, the commonly cultivated potato (*Solanum tuberosum*) belongs to the series *Tuberosa,* one of 19 series of the subsection *Potatoe,* which comprises 225 wild tuber-bearing species (Hawkes, 1992). The potato was introduced into Europe on two occasions, first around 1570 into Spain and subsequently between 1588 and 1593 into England (Hawkes, 1992). By the end of the 18th century the potato had become a major crop in Europe, particularly in Germany and western England. Potato cultivation continued to spread throughout the world during the first four decades of the 19th century. Nowadays the potato (*Solanum tuberosum* ssp. *tuberosum*) is one of the world´s major crops with an annual production of up to 311 million metric tons (*Mt*) in 2003. According to recent FAO statistics (FAOSTAT, June 2003, http://apps.fao.org), the potato ranks 5th in worldwide food production after sugar cane (1333 million *Mt*), maize (638 million *Mt*), rice (589 million *Mt*) and wheat (556 million *Mt*) on the list of crop species. Europe is the largest potato producer in the world producing 130.8 million *Mt* (42.1 %). The second largest producer is Asia (122.8 million *Mt*, 39.4 %) followed by North America (28.6 million *Mt*, 9.2 %), Africa (12.5 million *Mt*, 4.0 %) and South America (14.1 million *Mt*, 4.5 %). However, with the break down of the former USSR, China with 66.8 million *Mt* (21.5 %) is the largest producing country in the world followed by Russia FED (34 million *Mt*, 11.2 %), India (22 million *Mt*, 7.1 %), USA (19.8 million *Mt*, 6.3 %), Ukraine (18.5 million *Mt,* 5.9 %), Poland (13.7 million *Mt*, 4.4 %) and Germany (9.8 million *Mt,* 3.2 %). In Europe, Germany ranks second in potato production and in 2003, potatoes were planted on 283,624 ha. From the utility side, more than half of total

worldwide potato production (ca. 63 %) is used for table consumption, 13.8 % for livestock, 11.6 % for seed and 4 % for processing.

1.2 Potato genetics

The wild relatives of the cultivated potato exist in a polyploid series from diploid to hexaploid, whereas the commonly cultivated potato (*Solanum tuberosum*) is an extreme heterozygous autotetraploid (2n=4x=48) having 48 chromosomes and 4 genome complements (Hawkes, 1994). Like the tomato, another representative of the *Solanaceae* tribe, a complement of the potato genome consists of 12 chromosomes having ca. 0.7 pg (1 x 10^9 base pairs) of DNA (Dárcy, 1976; Ganal *et al*., 1990). In comparison *Arabidopsis thaliana,* the first higher plant with a completely sequenced genome, is known to have 120 x 10^6 base pairs. In classical potato breeding, selection for important polygenic traits should be orientated towards maximizing heterozygosity. For example, the vigor in autotetraploids is highly dependent on intra-locus diversity (Bingham, 1983; Hermsen, 1984; Bonierbale *et al*., 1993). The allelic structure within each of the loci for yield in these cultivars is unknown but expected to be highly heterozygous due to selection pressure for good performance (Tai & De Jong, 1997). In potato, selection is performed by evaluation of more than 50 traits with respect to different utilization purposes and it takes 10-15 years to generate a new variety. The current plant breeding efforts based on DNA markers can advance potato breeding and genetics, however, the complexity of autotetraploids is a stumbling block for genetic studies of potatoes. Chase (1963) proposed an analytical breeding scheme to reduce the chromosome number from autotetraploid to diploid level and dihaploids were first induced from tetraploids through a parthenogenic process (Hougas & Peloquin, 1958; Hermsen & Verdenius, 1973). Monohaploids were induced by this same process (van Breukelen *et al*., 1975, 1977; Jacobsen & Sopory, 1978). Also an androgenic process could be used for inducing dihaploids (Foroughi-Wehr *et al*., 1977; Sopory *et al*., 1978; Wenzel & Uhrig, 1981) and monohaploids (Irikura & Sakaguchi, 1972; Irikura, 1975; Foroughi-Wehr *et al*., 1977; Jacobsen &

Sopory, 1978; Sopory *et al*., 1978). Dihaploid materials derived from parthenogenesis were used for resistance tests (Cockerham, 1970) and protoplast fusion (Austin *et al*., 1985). Valkonen *et al*. (1998) reported leaf-drop symptom of virus-infected potato using anther culture-derived haploids. Recently anther culture-derived monoploids have been the basic materials for linkage analysis (Tai *et al*., 2000). The dihaploids induced through a parthenogenic or androgenic process are products of 6 different gamete combinations for one chromosome number when the recombination did not happen in meiosis (Fig. 1).

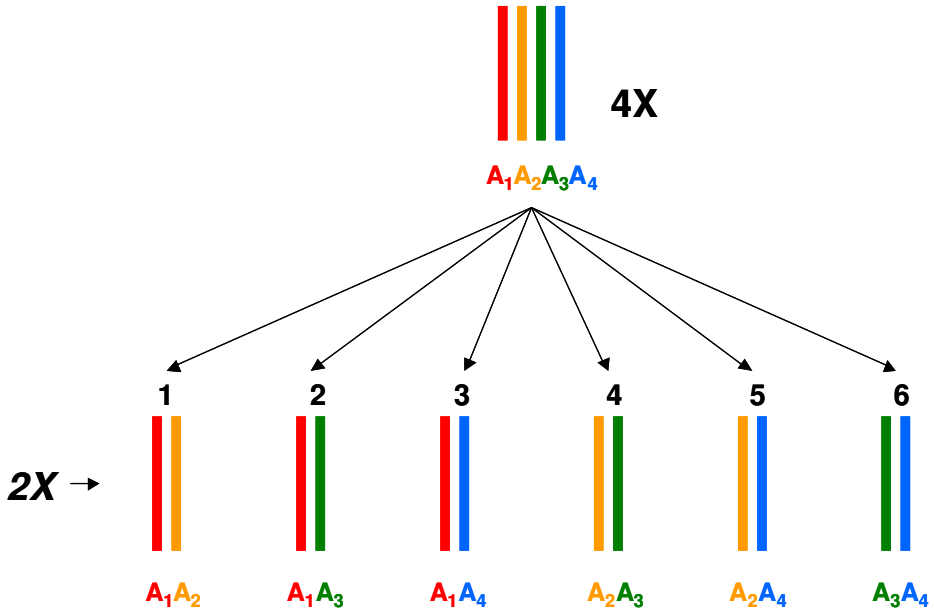


Fig. 1: The scheme of gamete segregation through a parthenogenic or androgenic process in autotetraploid potatoes

When a single locus is considered with two alleles *A/a* and *A* is dominant to *a*, there are five possible genotypes in tetraploids: quadruplex (*AAAA*), triplex (*AAAa*), duplex (*AAaa*), simplex (*Aaaa*) and nulliplex (*aaaa*). During meiosis, the most representative duplex (*AAaa*) or simplex (*Aaaa*) genotypes segregate 5 to 1 (*A-:aa*) or 1 to 1 (*Aa:aa*) if recombination events are neglected. Accordingly, dihaploid populations monitor these segregation ratios. Therefore, genetic analysis is much easier than monitoring tetraploid populations composing the total combination of 8 alleles.

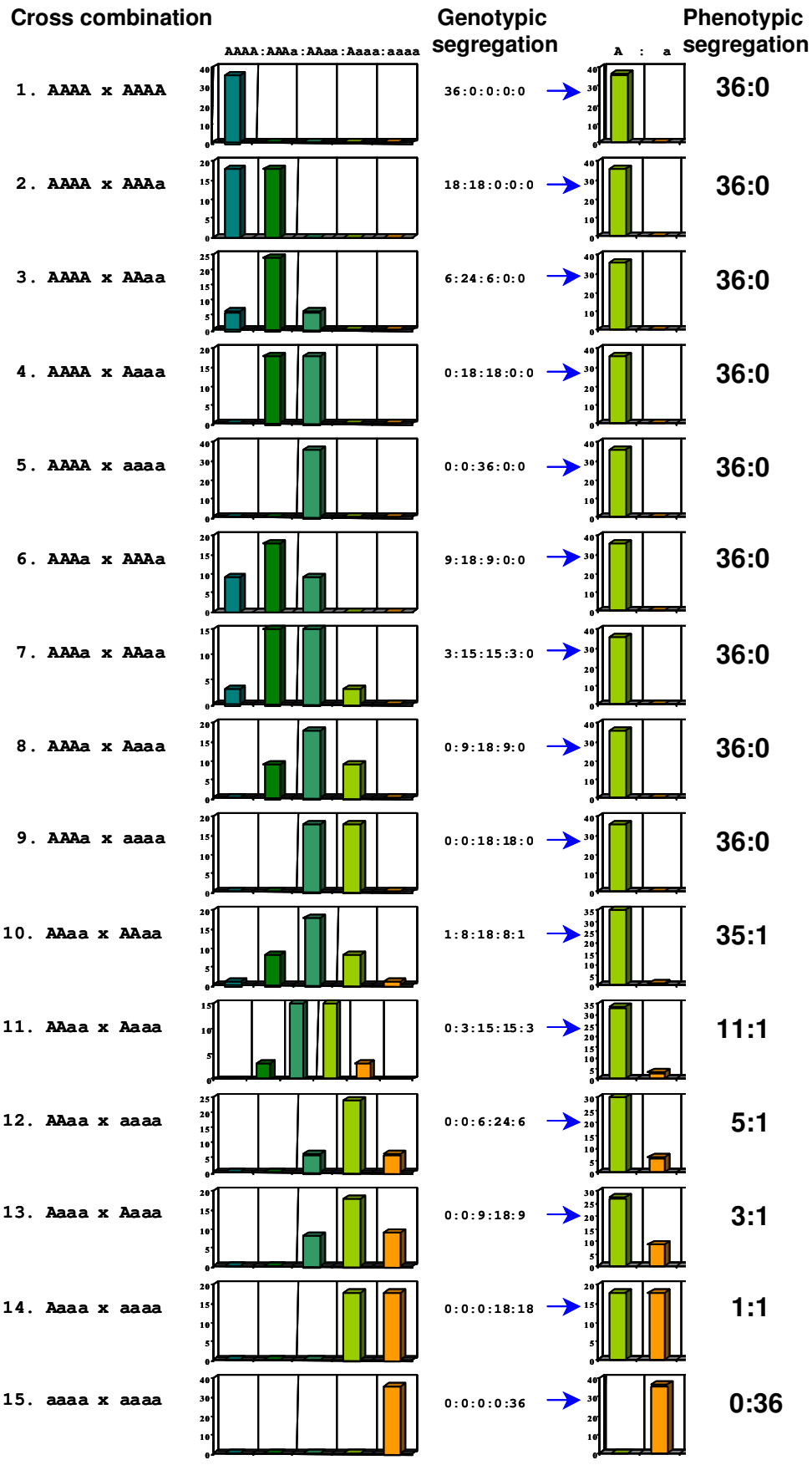


Fig. 2: Expected genotypic and phenotypic segregating ratio in tetraploid F_1 population
A represents one allele and is dominant to *a*

Under the same assumption, 15 different genotype-crossing combinations and genotypic segregation ratios in tetraploid F_1 progenies are possible (Fig. 2). In addition, there are 3 more phenotypic segregation types (35 to 1, 11 to 1 and 3 to1).

1.3 Molecular marker development and mapping in potatoes

Selection of new and superior genetic variants is the key to success in plant breeding programs. The utility of new tools is required to assist the screening and selection of new genotypes (Tanksley *et al*., 1989). In general, there are 3 types of markers available. Phenotypic markers based on visible characters, biochemical markers based on the detection of enzymes (e.g. isozymes) and molecular markers based on DNA. Phenotypic and biochemical markers are answers to gene expression that can be affected by the environment or stages of development and this analysis is time-consuming. On the other hand, DNA based molecular markers monitor the genotype directly, are not affected by the environment or differentiation, and can be applied in the seedling stage with representative Restriction Fragment Lengths Polymorphism (RFLP) analysis or Polymerase Chain Reaction (PCR) analysis.

Botstein *et al*., (1980) proposed the RFLP method for mapping genes that laid the groundwork for the Human Genome Project. Since this development, this technique has been applied to many other genetic fields including plant breeding. The first molecular map of the potato was constructed using tomato RFLP markers between the potato and tomato based on 1 to 1 segregating polymorphism from only one parent (Bonierbale *et al*., 1988). The genetic map of the potato was constructed per chromosome composition of three linkage groups. Two linkage groups were derived from each female or male parent with 1 to 1 segregating markers (Gebhardt *et al*., 1989) and one linkage group was constructed with 3 to 1 segregating markers derived from both parents (Gebhardt & Salamini, 1992). Gebhardt *et al*. (1991) pointed out that the potato genome is around 90 % similar to the tomato genome by identifying 12 linkage groups discovering 1000 cM (1Mb/cM) of potato compared to 750kb/cM in

tomato (Tanksley *et al.,* 1992). Jacobs *et al*. (1995) constructed separate maps for female and male parents with molecular markers, including transposons, and classical markers. The total length of the combined map covered 1120 cM. RFLP analysis was also used for mapping of various potato disease resistance loci, i.e. is for resistance to potato virus X, potato virus Y, *Phytophthora infestans* and nematodes (Ritter *et al*., 1991; Hämäläinen *et al*., 1997; Leonard-Schippers *et al*., 1992; Ballvora *et al*., 1995; Bonierbale *et al*., 1994).

The RFLP technique is expensive, laborious and time-consuming compared to the easier PCR analysis. Random Amplification of Polymorphic DNA (RAPD) analysis is a PCR based technique that produces polymorphism using a non-specific random 10-mer primer and *Taq-polymerase* (Williams *et al.,* 1990). However, low reproduction of RAPDs is pointed out as one disadvantage. RAPD analysis combined with Bulked Segregant Analysis (BSA) (Michelmore *et al*., 1991) has been used to identify DNA sequences linked to traits of interest in potatoes (Jacobs *et al*., 1996; Hosaka *et al*., 2001; Arnedo-Andrés *et al*., 2002; Bryan *et al*., 2002), in rice (Huang *et al*., 2003) and in wheat (Najimi *et al*., 2002). This method is a very suitable and rapid strategy for monogenic qualitative traits. For example in BSA, the DNA of ten plants will be mixed and bulked into one pool and all alleles should be represented. Two bulked pools differing for one trait will differ only at the locus harboring that trait.

Simple sequence repeats (SSR) markers are used for generation of identical linkage maps in crop plants by application of primers specific to the sequences flanking the microsatellite of the chromosome. Characterization and mapping of SSR loci pointed out their suitability for linkage analysis and agronomic trait selection (Yu & Pauls, 1993; Zhang *et al*., 1994). SSR are short, randomly repeated DNA motifs like (AC)n, or (ATT)n and are highly abundant in animal and plant genomes (Hamada *et al.,* 1982; Tautz & Renz, 1984). SSR fragments are mainly used for identification of germplasm assessment and variation and were aligned to previously published RFLP anchor markers in potatoes (Milbourne *et al*., 1998).

Amplified fragment-length polymorphism (AFLP™) analysis was developed by Vos *et al*. (1995). AFLP analysis combines RFLP and RAPD analysis based on selective PCR amplifications of small restriction fragments (80-400 bp) of

genomic DNA. AFLP fragments are normally dominant markers. AFLP analysis is very efficient for mapping whole genomes because many polymorphisms are produced. The chromosomal localization of AFLP markers was mapped together with RFLP and isozyme markers (Van Eck *et al.*, 1995). Recently, an Ultra-High Density Map (UHD-Map) was constructed by five research laboratories, which reside in Scotland, Spain, France and The Netherlands (http://www.dpw.wau.nl/uhd/).

Some valuable monogenic inherited agronomic traits of potatoes have already been mapped: Yellow flesh color (*Y*) on chromosome III (Bonierbale *et al.*, 1988), purple skin color (*PSC*) on chromosome X (Gebhardt *et al.*, 1989), flower color loci for red (*D*) and blue (*P*) anthocyanins on chromosome II and XI and tuber shape (*Ro*) on chromosome X (Van Eck *et al.*, 1994). Resistance genes for several diseases and pathogens have also been mapped: resistance gene *Gro*1 to the nematode *Globodora rostochiensis* on chromosome VII (Ballvora *et al.*, 1995), gene *GroV*1 to the same nematode derived from the wild species *Solanum vernei* on chromosome V (Jacobs *et al.*, 1996), the genes *Rx1* and *Rx2* (extreme resistance to potato virus X) on chromosome XII and V (Ritter *et al.*, 1991), the gene Nx_{phu} that controls hypersensitive resistance to potato virus X in *Solanum phureja* IvP35, was mapped on chromosome IX (Tommiska *et al.*, 1998) and the resistance gene to late blight (*Phytophthora infestans*) originating from *S. bulbocastanum* was mapped on chromosome VIII (Naess *et al.*, 2000).

The phenotypic expression of characters showing continuous or quantitative variation is controlled by the environment and by more than one gene. The loci where these genes are located in the genome are called "quantitative trait loci" (QTL) according to Gelderman (1975). Most of QTL mapping in potatoes initially proceeded in diploids to avoid the complexities of tetrasomic inheritance caused by greater deviations in the estimates of the heterozygous parameters as opposed to estimates of the homozygous parameters (Weller, 1987). The simplest way is to analyze the quantitative data using one marker at the time in a one-way analysis of variance with marker genotypes as classes, present and absent. This QTL analysis does not require a complete linkage map (Keim *et al.*, 1990). Simple regression analysis is to fit flanking marker models by multiple regression (Haley & Knott, 1992). Genetic expectations are calculated

for each marker genotype. The regression results are very similar to the maximum-likelihood method and therefore PROC REG programs (Regression analysis) in Statistical Analysis System (SAS) are widely used for estimating quantitative trait locus parameters and for testing the hypotheses about those parameters (Knapp & Bridges, 1990). Lander & Botstein (1989) developed interval mapping, a single-QTL model, with a maximum likelihood or logarithm of odds (LOD) threshold for the effect of a null hypothetical QTL at the position of each marker locus and at regular intervals between the marker loci. Interval mapping is a better approach for finding linkage between a QTL and marker genes. However, one of the factors that weakens interval mapping is fitting a model for a QTL at only one location so that the effects of additional QTL will contribute to sampling variance. The ideal solution would be to fit a model that contains the effects of all QTL like the multiple-QTL models (MQM) mapping method, developed by Jansen (1994) and Jansen & Stam (1994).

Quantitatively inherited valuable agronomic traits in potatoes have been analyzed by using various molecular markers and maps. In most cases, inter-diploid populations were used for analysis of resistance loci to nematodes (Kreike *et al*., 1993, 1994; Jacobs *et al*., 1996; Rouppe van der Voort *et al*., 1997, 1998, 2000), late blight (Leonards-Schippers *et al*., 1992, 1994; El-Kharbotly *et al*., 1994, 1996; van der Lee *et al*., 2001; Visker *et al*., 2003) and soft rot (Zimnoch-Guzowska *et al*., 2000). In some cases, however, tetraploid populations were used for analysis of resistance loci to nematode and late blight (Bradshaw *et al*., 1998; Li *et al*., 1998; Meyer *et al*., 1998). QTL for affecting tuber yield or tuber starch content were also reported (Schäfer-Pregl *et al*., 1998). Since the *Arabidopsis* genome project succeeded in complete, sequence data were applied to candidate genes related with resistance mechanism or specific metabolism in crops. A molecular function map was constructed by combining previous QTL maps and sequence information of carbohydrate metabolism and transport based on the sequence data bank of potatoes (Chen *et al*., 2001).

1.4 Extreme resistance to PVY (*Ry$_{sto}$*)

Potato virus Y (PVY) is one of the most important viruses in *Solanaceae* (tomato, tobacco, pepper) and can reduce potato production up to 80% (De Bokx & Huttinga, 1981). PVY is also wide spread and in some parts of Europe it is more common than the Potato Leaf Roll Virus (PLRV). The genome of PVY is composed of 9704 nucleotides (Robaglia *et al*., 1989) and belongs to the genus *Potyvirus* as well as potato virus *A* (PVA) and potato virus *V* (PVV). Potyviruses have mono-partite single-stranded, positive-sense RNA genomes and are with approximately 180 virus strains the largest genus of plant viruses (Hooker, 1981; Shukla *et al*., 1994). There are three well described PVY strain groups: PVY^{O} (common strains), PVY^{N} (necrotic strains) and PVY^{C} (stipple streak strains) according to Jones (1990) and Świeżyński (1994). In general virus infections give rise to serious problems in vegetative propagated crops like potatoes because of virus manifestation through all subsequent vegetative generations. In the last 10 years PVY has caused severe damage in potato seed production. In addition to the wide-spread PVY strains 0, N, which are already difficult to overcome by the use of insecticides and removal of infected plants, a new strain (PVY^{-NTN}) causing big necrotic rings on the tubers is presently attacking potatoes throughout Germany (Weidemann, 1993) and affects also the ware potato market.

There are two resistance mechanisms in plant cells against invading viruses. The first resistance mechanism can be characterized by the lacking of infection (immunity) or by strong reduction of virus replication in infected cells. The second resistance mechanism inhibits virus spread from cell-to-cell and through the vascular system (Ponz & Bruening, 1986; Valkonen & Somersalo, 1996; Bendahmane *et al*., 1999). Two major types of mono-genetically inherited resistance genes to PVY are known in cultivated and wild potato species (*Solanum* spp.), namely hypersensitive resistance (*Ny*) and extreme resistance (*Ry*) also designated as immunity (Ross, 1986). The hypersensitivity reaction inhibits virus movement and belongs to the second group of resistance but does not provide a total defense against virus Y.

Immunity or extreme resistance to potato virus Y (*Ry*) was first reported by Cockerham (1943) in *Solanum stoloniferum* Schlechtd., which is a tetraploid (2n=4x=48) Mexican wild potato species, that belongs to *Longipedicellata* Buk. and is very polymorphic with locally abundant forms differing in degree of leaf dissection and flower color from white to purple (Cockerham, 1970; Hawkes, 1992). Seven different phenotypic responses were classified for the combined reactions of *S. stoloniferum* to virus Y and to virus A:

Phenotype 1: No visible reaction to sap transfers of either PVY or PVA

Phenotype 2: No visible reaction to sap transfers of PVY, while sap inoculations with PVA induce local lesions on the inoculated leaves

Phenotype 3: No visible reaction to sap transfers of PVY, while sap inoculations with PVA induce heavy necrosis and symptoms of necrotic mosaic in the plant

Phenotype 4: Sap transfers with PVY cause local lesions and frequently lethal top necrosis, while sap and graft transfers with PVA produce non-lethal necrosis symptoms

Phenotype 5: Necrotic reactions to PVY similar to those in phenotype 4. PVA induces very mild symptoms and the virus is recoverable

Phenotype 6: Completely susceptible to PVY. Toward PVA this phenotype is similar to phenotype 3 and phenotype 4, exhibiting non-lethal rusty necrosis

Phenotype 7: Plants are completely susceptible to both PVY and PVA.

Comprehensive, extreme resistance is present only among phenotype 1 and the wild types CPC 9, CPC 28.4 and PI 160226 belong to this phenotype. This trait is controlled by a single dominant resistance gene Ry_{sto} that has been introgressed into *Solanum tuberosum* (Ross, 1952; 1958). Ross (1952) reported the practical importance of the *Ry*-mediated resistance because it is effective against all strains of PVY. In addition, Barker (1997) reported that Ry_{sto} confers resistance to PVA and PVV. Plants expressing *Ry* remain symptomless, except for limited necrosis developed in systemically infected leaves from a few genotypes following graft-inoculation. No PVY could be detected with ELISA in inoculated plants expressing *Ry* (Ross, 1986). Ross described approximately 20 cultivars carrying the gene *Ry* for extreme resistance to PVA and PVY derived from *S. stoloniferum*.

Extreme resistance to PVY has also been found in *S. tuberosum* spp. *andigena* (Muňoz *et al*., 1975; Galvez & Brown, 1980). However, Ry_{adg} confers only resistance to PVY and not to PVA. The International Potato Center (CIP) tried to produce breeding lines having triplex or quadruplex immunity (Ry_{adg}) according to Mendoza *et al*. (1986).

Cockerham (1970) reported a third gene source for extreme resistance to PVY from *S. hougasii* (6x). The gene Ry_{hou} controlled extreme resistance to all tested strains of PVY and PVA. Other simply inherited resistances to PVY were found in *S. chacoense* (2x), *S. microdontum* (2x), *S. demissum* (6x). Celebi-Toprak *et al*. (2002) reported hypersensititive resistance (Ny_{tbr}) to PVY in *S. tuberosum* on chromosome IV, whereas, intensive studies on mapping of PVY resistance genes (Ry_{sto} and Ry_{adg}) have been reported (Solomon-Blackburn & Barker, 2001) and markers for them were found on chromosome XI (Table 1).

Table 1: PVY resistance genes mapped in potatoes

Gene	Source	Chromosome	Position	Reference
Ry_{sto}	I-1039	XI	Between markers GP125 and CT182	Brigneti *et al*. (1997)
Ry_{adg}	Diploid 2x(v-2)7 with *adg* in pedigree	XI	Between markers GP125 and CT182	Hämäläinen *et al*. (1997, 1998)
Ra_{adg}	Diploid 2x(v-2)7 with *adg* in pedigree as Ry_{adg}	XI	6.8 cM distal to Ry_{adg}	Hämäläinen *et al*. (1998)

Valkonen (1994) has found evidence for two resistance genes (Ry_{adg} and Ny_{adg}) in *S. tuberosum* subsp. *andigena* and his group also detected a virus resistance gene, namely the gene Ra_{adg} for resistance to potato virus A, located only 6.8 cM away from Ry_{adg} towards the distal end of chromosome XI (Table 1). SCAR markers were developed for the PVY resistance gene Ry_{adg} by Kasai *et al*. (2000). Also the Ry_{sto} gene was reported to be localized on chromosome XI (Brigneti *et al*., 1997), however, the use of this region could not be used for selection.

1.5 Chip quality after long term storage at 4°C

The dry matter (DM) content is a very important factor for the quality of both boiled and fried potato products, because the weight of the processed product depends directly on the amount of DM present per quantitative unit of fresh potatoes (Burton *et al.,* 1992). Since 60-80% of the DM consists of starch, there is a major correlation between DM content and the starch content of the tuber (Burton *et al.,* 1992). Thus a defined high starch content of tuber is one of the most important selection criteria for good processing suitability of genotypes and also an agronomically important quantitative character that is controlled by genetic factors and by environmental conditions. Starch content and DM content are known for relatively low genotypes-environment interaction (Maris, 1962, 1969; Killick & Simmonds, 1974) and thus depend on cultivars. Optimized cultivars for chipping processing are required to have starch content between 16 % to 19 % in contrast to 17 to 18 % for French fries. Tubers with low starch content absorb more oil than ones having high starch content (Smith, 1987) whereas high starch content can lead to crispy injury inside the oral cavity. In practice, starch content of tubers is estimated by measuring specific gravity as the weight difference between a sample of tubers in air and the same sample under water (Von Scheele *et al*., 1937).

Starch accumulates in tubers (sink) following the translocation of sucrose from the leaf (source) via phloem vessels (Fig. 3). In tubers, sucrose is immobilized by conversion to starch in amyloplasts by starch synthase (Nr. 15, Fig. 3). During the storage period, starch in the tuber is degraded to reducing sugars (glucose, fructose) and sucrose dependent on various factors like sprouting, storage temperature and tuber maturity. Duplessis *et al*. (1996) reviewed four main types of sugar accumulation and sweetening in potato tubers. First is senescent sweetening after storage. The second type is sugar level increase associated with rapid sprouting. The third type is sweetening due to storage of physiologically immature potato tubers. The fourth type is an accumulation of both reducing sugars and sucrose when tubers are subjected to chilling temperatures, a phenomenon known as cold sweetening (Burton, 1969).

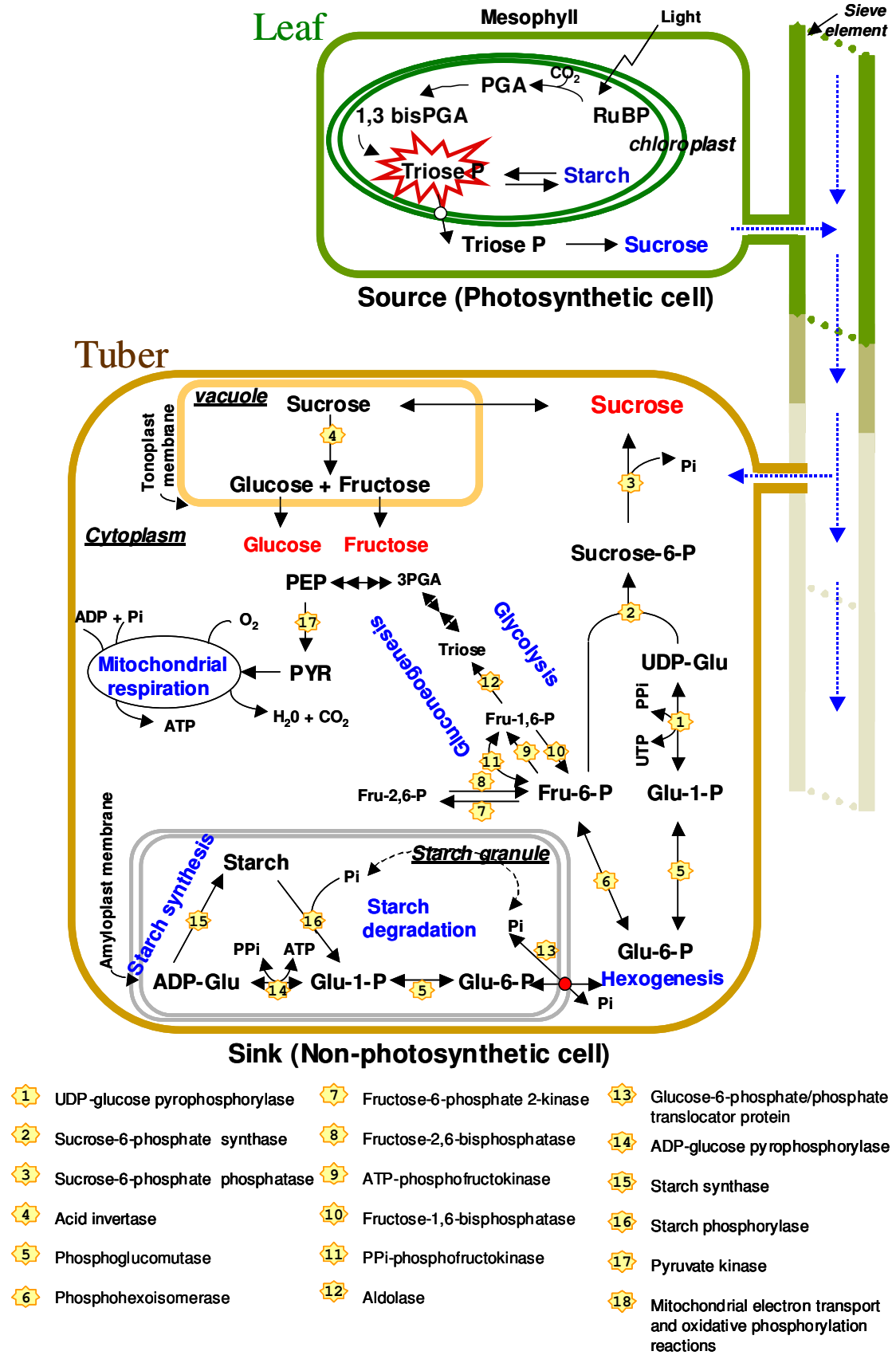


Fig. 3: Scheme of carbon movement from leaves (photosynthetic cell) to potato tubers (non-photosynthetic cell) and related carbohydrate pathways (Morrell & Ap Rees, 1986; Sowokinos, 2001)

PGA	:	Phosphoglycerate	**Glu-1-P**	:	Glucose-1-phosphate
1,3 bis PGA	:	1,3 bis-phosphoglycerate	**Glu-6-P**	:	Glucose-6-phosphate
Triose P	:	Triose-phosphates	**UTP**	:	Uridine triphosphate
Pi	:	Phosphate	**UDP-Glu**	:	Uridine diphosphate glucose
ADP	:	Adenosine diphosphate	**Sucrose-6-P**	:	Sucrose-6-phosphate
ATP	:	Adenosine triphosphate	**Fru-6-P**	:	Fructose-6-phosphate
PYR	:	Pyruvate	**Fru-1,6-P**	:	Fructose-1,6-phosphate
PEP	:	Phosphoenolpyruvate	**Fru-2,6-P**	:	Fructose-2,6-phosphate
ADP-Glu	:	Adenin diphosphate glucose	**PPi**	:	pyrophosphate
RuBP	:	Ribulose 1,5-bis-phosphate (pentose)	**3PGA**	:	3-phosphoglycerate

Fig. 3 continued

This low temperature sweetening in tubers was first reported by Müller-Thurgau in 1882 (Zrenner *et al*., 1996). Low temperature accelerates the accumulation of reducing sugars as a result of the degradation of starch and conversion of sucrose to glucose and fructose catalyzed by invertase. These accumulated reducing sugars in tubers are the major problem for the potato processing industry resulting in bitter-tasting, dark colored and therefore unacceptable processed products. This dark browning is called a non-enzymatic Maillard reaction (Talburt *et al*., 1975) caused by an interaction between free aldehyde groups of hexose reducing sugars (glucose and fructose) and free α-amino groups of amino acids and tuber proteins of nitrogenous compounds during high temperature processing. The acceptability of potatoes for processing into chip is largely dependent on the color of the end product. Therefore, the level of reducing sugars is a critical parameter. An ideal reducing sugar content should be in general below 100 mg per 100 g of tuber fresh weight (0.1 %), whereas for French fries, it is required to be below 0.25 % and levels higher than 0.33 % are unacceptable (Dale & Mackay, 1994; Davies & Viola, 1992). Isherwood (1973) reported an inter-conversion between starch and sugar. It was found that the starch-carbon is the source of accumulated reducing sugars when changing the storage temperature back to 10°C after cold storage at 2°C. During cold-induced sweetening in stored potatoes, starch degradation occurs primarily through the action of starch phosphorylase. Reducing sugars were accumulated through the enzymatic reactions illustrated in Figure 3 (Morrell & Ap Rees, 1986; Sowokinos, 2001). Sowokinos *et al*. (2000) reviewed the complex sweetening metabolism by cold stress. Starch, total solids and sucrose decreased markedly while glucose content increased 15-fold. Basal acid

invertase activity increased 7-fold during storage. Sowokinos *et al*. (1993) reported the correlation (r=0.92) between UGPase activity and the amount of accumulated glucose, the product of sucrose hydrolysis. The cold-induced hexose accumulation is caused by an imbalance between starch breakdown and glycolytic activity, and the cold-lability of several enzymes from glycolysis such as phosphofructokinase and pyruvate kinase (Pollock & Ap Rees, 1975; Dixon & Ap Rees, 1981).

Considering the negative consequences of sweetening, the processing industry relies on the use of chemical sprout suppressants, e.g. N-(3-chlorophenyl) carbamate (Chloropropham), especially for long storage periods at approximately 8-10°C to delay sprouting. The chemical most commonly used as a sprout suppressant is CIPC (isopropyl-N-chlorophenylcarbamate) by interfering with spindle formation during cell division. Marth & Schultz first reported the use as a potato sprout inhibitor in 1952. Effects of CIPC are internal sprouting inhibition during warm temperature storage (Ewing *et al*., 1968; Ewing, 1974) and reduction of respiration (Boe *et al*., 1974). However, it has been reported that the utility of sprouting inhibitor is not connected with chip quality (Sherman & Ewing, 1982). According to Kaplan (2001), more than $2.5 billion are required for sprouting control in the U.S.A., and the use of CIPC is still growing. On the other hand, the utility of CIPC is still a hot issue of debate due to concerns about its carcinogenicity. There is concern about residues of CIPC on tuber-surfaces that may be midly toxic as well as the possible effects of environmental pollution produced by the waste of the processing industry. Lang (1992) reported that potato peels contain chemical residues and the peels of dry or moist-cooked potatoes retain up to 20 times more sprout inhibitor residues than the pith. In the United States, CIPC is currently the only registered post harvest sprout inhibitor for potatoes and is used under security during storage, whereas the use of CIPC is already partially banned in Europe. In Germany the use of CIPC, i.e. Neostop, is allowed to inhibit sprouting related with low respiration during warm storage (8°C to 10°C) and to reduce the accumulation of reducing sugars at low temperatures. In some Scandinavian countries the use of CIPC was forbidden several years ago and Denmark has prohibited the use of these chemicals since fall 1999 (Brouwer & Bijman, 2001).

A report from Sweden in April 2002 on the detection of acrylamide, a compound assumed to be carcinogenic, has alarmed the potato industry and consumers alike. The acrylamide may form in high concentrations when certain foods, not only potato products such as potato chip and French fries, but also breakfast cereals, and more generally starch containing foods, are cooked at high temperatures. On the other hand, Pelucchi *et al*. (2003) reported a data analysis from cancer patients that provided reassuring evidence for the lack of a link between the consumption of fried/baked potatoes and an increased cancer risk. The data indicate that it is too early to come to a conclusion on this issue. Potato chip and French fries has been the representative subject of study as a modeling system for the formation of acrylamide (Biedermann *et al*., 2002; Biedermann-Brem *et al*., 2003; Chuda *et al*., 2003; Noti *et al.,* 2003; Pelucchi *et al*., 2003; Pollien *et al*., 2003). Zyzak *et al*. (2003) reported the formation mechanism of acrylamide from the reaction of amino acid asparagines and a carbonyl-containing compound at typical cooking temperatures. Amino acid asparagines also formed especially in potato tubers stored at low temperature (Brierley *et al*., 1997). Minimizing the potential of acrylamide formation will also reduce the browning of some potato products for which the darker color and ensuring flavors are desired. It has been recommended not to store the potato below 8℃ (Biedermann-Brem *et al*., 2003; Noti *et al.,* 2003). However, this connection creates a dilemma with the use of chemical sprout inhibitor (CIPC) in warm storage or the potential of acrylamide formation in cold storage. Amrein *et al*. (2003) showed large differences in the potential of acrylamide formation in various cultivars primarily related to the sugar content of these cultivars (Loiselle *et al*., 1990; Hanneman, 1996). Acrylamide formation can thus be limited by selecting cultivars with low concentrations of reducing sugars.

As food safety and environmental concerns rise, new storage forms will be attempted that do not involve the use of chemical sprout inhibitors. Therefore, the breeding of new cultivars with resistance to cold sweetening, reduced sprouting and minimized accumulation of reducing sugars is required. Low temperature storage is an alternative natural control with many benefits. Under low temperatures it is easier to keep the high humid atmosphere required for minimizing evaporative loss, reduction of senescent sweetening and shrinkage

of losses due to storage rot and other pathogen problems (Burton, 1969; Duplessis *et al*., 1996). Furthermore, cold storage could minimize dry matter reduction through reduced respiration and thereby increase the marketing window (Davies & Viola, 1992). The identification of cultivars with good chip quality after storage at 4°C will allow growers to reduce storage losses without the use of post harvest agrochemicals and reconditioning before processing. This indicates that the breeding of cultivars having low reducing sugars after cold storage stands out as an alternative solution.

The effects of cold on potato metabolism are likely to be very complex. Two different cDNAs of potato tuber UGPase have been cloned and sequenced (Katsube *et al*., 1990; Spychalla *et al*., 1994). UGPase was reported to be a single-copy gene in the haploid genome (Borovkov *et al*., 1996). Using antisense constructs, UGPase expression was partially reduced (30 to 50%) and lower levels of sucrose were yielded in transgenic potatoes (Borovkov *et al*., 1996). The cDNA of a cold-inducible soluble acid invertase was isolated from cv. “Desiree” (Zrenner *et al*., 1996). Antisense inhibition of soluble acid invertase activity reduces the accumulation of hexose but increases the sucrose content during cold storage of potato tubers. Greiner *et al.,* (1999) reported that hexose accumulation was significantly reduced in transgenic tubers having cold-inducible vacuolar invertase in antisense orientation. Thill & Peloquin (1994) suggested a three key loci hypothesis in the inheritance of chip color at the 24 chromosome level. This three key loci hypothesis is the simplest model for good chipping results when there should be at least one dominant allele at each of the three loci. Poor chipping quality occurred when at least one locus had two recessive alleles, regardless of the allelic constitution of the remaining two loci. Douches & Freyre (1994) reported six QTL for chip color based on one-way analysis of variance (ANOVA) and additive effects contribute to a significant portion of the genetic variance for chip color. The sugar content of potato tubers is reported as a quantitative trait with heritability values ranging from very high (r=0.91) to intermediate (r=0.47-0.63) published by Grassert *et al*. (1984) and Pereira *et al*. (1994). The constructed plant molecular-function map for the potato (Chen *et al*., 2001) reveals also genes responsible for quantitative traits of the carbohydrate metabolism and transport at the molecular

level. QTL for cold sweetening were analyzed using candidate's genes (Menéndez *et al*., 2002).

1.6 Aims of this dissertation

In this dissertation, two important potato breeding traits, extreme resistance to PVY (Ry_{sto}) and chip quality after long term storage at 4°C, a qualitative and a quantitatively inherited trait, are analyzed by molecular marker technologies. In general, the aim of this work is to develop molecular markers for each of these traits and to evaluate the utility of these markers for marker assisted potato breeding programs.

Extreme resistance to PVY in German potato cultivars is based only on resistance genes from *S. stoloniferum*. Therefore, more research on the establishment of genetic markers for this resistance is necessary. In relation to this need, the aims of my research studies concerning extreme resistance to PVY can be summarized as follows:

- Establishment of genetic markers, which are related to the resistance gene(s) to potato virus Y (PVY) using a dihaploid population produced by anther culture from the PVY-immune cultivar "Assia" by AFLP analysis.
- Localization of the Ry_{sto} markers in the potato genome map to check the linkage to chromosome XI.
- Testing the Ry_{sto} markers in cultivated potatoes in order to determine the quality of the Ry_{sto} markers for selection of extreme resistant cultivars and breeding lines in further potato breeding programs.
- Confirmation of the Ry_{sto} markers by pedigree analysis of extreme resistant cultivars.
- Development of diagnostic PCR markers from AFLP markers and testing the applicability of these markers.

The cold temperature storage ability of potatoes is a hotly debated topic and is a quantitatively inherited trait. Nevertheless, some breeding success has resulted in lines and cultivars with good chip quality by reduced accumulation of

reducing sugars after long term storage at cold temperature: ND860-2 and ND2221-6 from U.S.A. (Ehlendeldt *et al*., 1989, 1990), “Artis”, “Sempra” and “Tessi” from Germany (Putz, 2001). Theses lines and cultivars have the advantage that they can be used directly for chipping without reconditioning. More research on the establishment of genetic markers for quantitatively inherited trait is necessary. The aims of my research studies concerning chip quality after long term storage at 4°C can be summarized as follows:

- Linkage group analysis by AFLP, RFLP and SSR analysis within a dihaploid population induced by crossing with *S. phureja* from the cultivar “Arits” having good phenotypes after long term storage at 4°C.
- Evaluation of phenotypic variance of starch content, reducing sugar content and chip color in these dihaploids.
- Establishment of QTL analysis for starch content, chip color and reducing sugar content in dihaploids.
- Localization of markers for starch content, chip color and reducing sugar content on the genetic maps of potato by molecular marker analysis.
- Analysis of marker segregation patterns in the dihaploid and tetraploid populations and evaluation of merged marker results.

2 Materials and methods

2.1 Plant material

2.1.1 Extreme resistance to PVY (*Ry$_{sto}$*)

2.1.1.1 Basic material

Basic material for the PVY-project was the cultivar “Assia” from the German breeding station Uniplanta-Saatzucht KG, Brunnen established in 1980. “Assia” is recorded with middle early-maturity and economic utility type and extreme resistance to PVY (Beschreibende Sortenliste, 1990). The immunity to PVY in “Assia” (*adg, acl, dms, sto, vrn*) was derived from *S. stoloniferum* according to pedigree data (Ross, 1986; Świeżyński *et al*., 1997).

2.1.1.2 Population for marker development

Through anther culture method, 124 plants were regenerated from microspores of “Assia” at the potato biotechnology department of the Bayerische Landesanstalt für Landwirtschaft from 1996 to 1997 (Schwarzfischer *et al*., 2002). 89 lines survived in the green house and were used for artificial potato virus Y infection tests. For resistance marker development, however, 10 mixoploid lines detected by flow cytomtery (Partec CA-II, Münster) and 13 homologous lines detected by identical AFLP-patterns were removed so that finally 57 different exactly dihaploid lines were used for identification and localization of resistance markers.

2.1.1.3 Cultivars for marker selection

In addition to the selected resistant and susceptible dihaploid lines according to PVY infection test and ELISA results, ten cultivated potatoes (“Assia”, “Bettina”, “Forelle”, “Ute”, “Kuras”, “Sibu”, “Petra”, “Tomba”, “Laura” and “Sempra”) were tested as control group. Among them “Bettina”, “Forelle” and “Ute” were registered as cultivars with PVY-immunity (Beschreibende Sortenliste 1979; 1982; 1988). “Kuras”, “Sibu”, “Petra” and “Tomba” were classified as highly

resistant to potato virus Y while “Laura” and “Sempra” were recorded with low resistance to PVY (Beschreibende Sortenliste, 2001).

2.1.1.4 Cultivars for marker proofing

Based on the marker selection results, the determined markers were tested in 110 potato varieties from Germany, The Netherlands and Poland summarized together with the results on page 56-57.

2.1.2 Chip color quality after long term cold storage

2.1.2.1 Basic material

Among 84 cultivated potatoes tested for cold chipping by Bundessortenamt (Beschreibende Sortenliste, 2002) only the cultivars “Artis”, “Sempra” and “Tessi” were recorded to have good chip quality (note 6, note 7, and note 6) after long term storage at 4°C. The chip color quality scale of Bundessortenamt is classified from note 1 (dark color) to note 9 (light color). The cultivars “Artis”, “Sempra” and “Tessi” were derived from the same crossing between breeding line UP 0.711/44 as the female parent and “Panda” as the male parent at the breeding station Uniplanta-Saatzucht KG.

2.1.2.2 Dihaploid population

Dihaploid lines from “Artis” were produced at the potato breeding department of the Bayerische Landesanstalt für Landwirtschaft (LfL) using the pollinator *S. phureja* IVP48 (2n=24) from 1996 to 1998. 137 berries were harvested from crossings (H1531) between “Artis” and *S. phureja* IVP48. In total, 2507 seeds were obtained among them 1376 seeds (54.9%) with the dominant red spot in the embryo (selection marker from *S. phureja*) were wasted, and 1131 seeds were established as *in vitro* culture. After flow cytometry analysis, 337 lines could be selected as dihaploid lines. However, in the first year, only 83 lines of dihaploid Artis potatoes could be successfully propagated in the green house and in the field. Therefore, in this study only the 83 dihaploid potato lines from Artis were used for molecular marker development, field tests and chip color tests after long term cold storage.

2.1.2.3 Tetraploid population

For application test and segregation studies of selected marker alleles from the dihaploid population to a tetraploid population, 100 tetraploid “Artis” lines were produced by crossing “Artis” as the female parent and the tetraploid breeding line 2899/9B as the male parent by the potato breeding department of the LfL in 1997.

2.2 General molecular marker analysis methods

2.2.1 Genomic DNA isolation

Total genomic DNA was extracted from *in vitro* cultured plants or field material with modified CTAB methods (Saghai-Maroof *et al*., 1984). Fresh plant material (6g) was finely ground in a pre-chilled (-20°C) mortar using liquid nitrogen. The resulting fine powder was transferred into a 50 ml Falcon tube containing 24 ml 2x-CTAB buffer. After vortex, the mixtures were incubated in a water-bath (60°C) with strong shaking for 1 hour. After incubation, the probes were cooled in ice water and 25 ml of a chloroform isoamylalcohol mixture (49:1) were added. Using an invert shaker (Reax2, Germany) the tubes were inverted for 30 minutes at 60 rpm. After centrifugation at 5000 rpm for 15 minutes (Sigma 1-15, Germany), cell debris, proteins and polysaccharides were collected on the bottom of the chloroform mixture phase. The supernatants were transferred into new 50 ml Falcon tubes containing 1µl of RN*ase* (10 mg/l) with a 5 ml pipette with cut tip and incubated at room temperature for at least 30 min or overnight. After RN*ase* treatment, chloroform extraction was repeated again for purification. Two-thirds volume of iso-propanol was added to the purified supernatant and the mixture was inverted gently for forming the DNA pellet. The probes were stored at –20°C for 1 hour for further condensing of the DNA pellet. DNA was hooked by a glass stick and put into 76 % ethanol containing 0.2 M sodium acetate. After centrifugation, the DNA was washed with 76 % ethanol containing 10 mM ammonium acetate. The DNA pellet was dried at room temperature and dissolved in TE buffer (pH 8.0). The DNA concentration was estimated in a 0.8 %

agarose gel by comparing with Lamda/*Hind*III marker. Finally, the concentration was adjusted to 500 ng/µl.

2.2.2 Amplified Fragment Length Polymorphism (AFLP™) analysis

2.2.2.1 General information

This non-radioactive AFLP was performed with small modifications according to Hartl & Seefelder (1998) with a modified fluorescent-labeled primer AFLP system method that is based on the "AFLP-Protocol for Public Release" version 2.0 from KeyGene Company, The Netherlands (Vos *et al*., 1995).

2.2.2.2 Restriction-ligation reactions

The genomic DNA (500 ng/µl) was diluted to 50 ng/µl. Around 250 ng of DNA were digested with 2.5 U of the restriction enzymes *Eco*RI or *Pst*I (rare cutter) and with 2.5 U of *Mse*I (frequent cutter) in reaction buffer NEB 2 (New England Biolabs, Frankfurt) with 0.1 mg/ml of BSA (New England Biolabs, Frankfurt) for 3 h at 37°C. Then 5 µl of ligation mixture containing 0.2 µM *Eco*RI-adapters or *Pst*I-adapters, 2 µM *Mse*I-adapters, 1 U T4 DNA-ligase, 1 mM ATP in reaction buffer (NEB 2) and 0.1 mg/ml of BSA were added. The reaction was continuously incubated at 37°C for 1 h (Table 2).

Table 2: Restriction and ligation reaction in AFLP assay

Restriction reaction			**Ligation reaction**		
Reagent	**Final concentration**	**Volume**	**Reagent**	**Final concentration**	**Volume**
50 ng/µl genomic DNA	250 ng	5.000 µl	T4 DNA ligase (4U/µl)	1.0 U	0.25 µl
*Eco*RI or *Pst*I (20 U/µl)	2.5 U	0.125 µl	10X BSA (1mg/ml)	0.1 mg/ml (1 x)	0.50 µl
			10 mM ATP	1.0 mM	0.50 µl
*Mse*I (10 U/µl)	2.5 U	0.250 µl	*Eco*RI adapter (5µM)	0.5 µM	0.50 µl
10X NEB2 buffer	1 X	2.000 µl	(or *Pst*I adaptor)		
10X BSA (1 mg/ml)	0.1 mg/ml (1 x)	2.000 µl	*Mse*I adapter (50µM)	5 µM	0.50 µl
H_2O		10.625 µl	10X NEB2 buffer	1 X	0.50 µl
			H_2O		2.25 µl
Total volume		20.00 µl	Total volume		5.00 µl

After ligation reaction, the reaction mixture was diluted to 100 µl with TE buffer containing 10 mM Tris-HCl, 0.1 mM EDTA (pH 8.0) and stored at –20°C. The

cutting site sequences of restriction enzymes and adaptor sequences are presented in Table 3.

Table 3: Restriction enzyme cutting sites and adapter sequences

Enzyme	Cutting site		Adapter	Adapter sequence
***Eco*RI**	5´-G↓AATTC-3´ 3´-CTTAA↑G-5´	6 bp / cohesive end	*Eco*RI-adapter	5´-CTCGTAGACTGCGTACC-3´ 3´-CATCTGACGCATGGTTAA-5´
***Pst*I**	5´-CTGCA↓G-3´ 3´-G↑ACGTC-5´	6 bp / cohesive end	*Pst*I-adapter	5´-CTCGTAGACTGCGTACATGCA-3´ 3´-CATCTGACGCATGT-5´
***Mse*I**	5´-T↓TAA-3´ 3´-AAT↑T-5´	4 bp / cohesive end	*Mse*I-adapter	5´-GACGATGAGTCCTGAG-3´ 3´-TACTCAGGACTCAT-5´

2.2.2.3 Pre-amplification and selective amplification

According to Vos *et al.* (1995), AFLP primers consist of three parts, a core sequence (CORE), an enzyme-specific sequence (ENZ) and a selective extension (EXT). The code following E (*Eco*RI), P (*Pst*I) or M (*Mse*I) indicating restriction enzymes, refers to additional selective nucleotides at the 3´-end of the primer (Table 4). At the pre-selective amplification step, primers with one selective base at each side, E+A/M+C and P+G/M+C were used.

Table 4: Selective and pre-selective amplification primers

	CORE ENZ EXT
*Eco*RI primer form	5´-GACTGCGTACC AATTC *NNN-3´
Pre-selective E+A	5´-GACTGCGTACC AATTC A-3´
*Pst*I primer form	5´-GACTGCGTAC ATGCA G *NNN-3´
Pre-selective P+G	5´-GACTGCGTAC ATGCA G G-3´
*Mse*I primer form	5´-GATGAGTCCTGAG TAA *NNN-3´
Pre-selective M+0	5´-GATGAGTCCTGAG TAA -3´
Pre-selective M+C	5´-GATGAGTCCTGAG TAA C-3´

*NNN indicates selective nucleotides

First, the diluted ligation reaction was amplified with pre-selective primers in a total volume of 20 µl as described in Table 5 using a Eurogentec PCR reaction kit (Eurogentec, Köln). The PCR profile started with an incubation step for 2 min at 72°C, followed by 20 cycles of 30s at 94°C, 1 min at 60°C and 2 min at 72°C. After amplification, the PCR products were diluted to 400 µl with de-ionized water.

Table 5: PCR assay for pre-selective and selective amplifications

Pre-selective amplification			**Selective amplification**		
Reagent	**Final concentration**	**Volume**	**Reagent**	**Final concentration**	**Volume**
Diluted restriction - ligation products		5.00 µl	Diluted pre-selective amplified products		3.00 µl
10x reaction buffer	1 X	2.00 µl	10x reaction buffer	1 X	1.00 µl
50 mM $MgCl_2$	1.5 mM	0.60 µl	50 mM $MgCl_2$	1.5 mM	0.30 µl
10 mM dNTP	0.4 mM	0.80 µl	10 mM dNTP	0.4 mM	0.40 µl
[1]Pre-selective primer pair (*Eco*RI+*Mse*I or *Pst*I+*Mse*I) each primer (10 µM)	each 0.3 µM	each 0.60 µl	[2]Selective primer pair (*Eco*RI+*Mse*I or *Pst*I+*Mse*I) each primer (10 µM)	each 0.3 µM	each 0.30 µl
Taq polymerase (5 U/µl)	0.50 U	0.10 µl	*Taq* polymerase (5 U/µl)	0.25 U	0.05 µl
H_2O		10.35 µl	H_2O		4.65 µl
		20.00 µl			10.00 µl

[1]Pre-selective primers are illustrated in Table 4. [2]Selective primers are illustrated in Table 6.

Selective amplification was conducted with three selective bases at the 3´end of both primers (Table 6).

Table 6: Code and sequence list of selective primers according to pre-selective amplifications

***Eco*RI selective primers** (´5→3´)

Pre-amplification: E+A

Code	CORE	ENZ	EXT
E+AAA	GACTGCGTACC	AATTC	AAA
E+AAC	GACTGCGTACC	AATTC	AAC
E+AAG	GACTGCGTACC	AATTC	AAG
E+AAT	GACTGCGTACC	AATTC	AAT
E+ACA	GACTGCGTACC	AATTC	ACA
E+ACC	GACTGCGTACC	AATTC	ACC
E+ACG	GACTGCGTACC	AATTC	ACG
E+ACT	GACTGCGTACC	AATTC	ACT
E+AGA	GACTGCGTACC	AATTC	AGA
E+AGC	GACTGCGTACC	AATTC	AGC
E+AGG	GACTGCGTACC	AATTC	AGG
E+AGT	GACTGCGTACC	AATTC	AGT
E+ATA	GACTGCGTACC	AATTC	ATA
E+ATC	GACTGCGTACC	AATTC	ATC
E+ATG	GACTGCGTACC	AATTC	ATG
E+ATT	GACTGCGTACC	AATTC	ATT

***Pst*I (*Sse*I) selective primers** (´5→3´)

Pre-amplification: P+G

Code	CORE	ENZ	EXT
P+GAA =S+AA	GACTGCGTACA	TGCAG	GAA
P+GAC =S+AC	GACTGCGTACA	TGCAG	GAC
P+GAG =S+AG	GACTGCGTACA	TGCAG	GAG
P+GAT =S+AT	GACTGCGTACA	TGCAG	GAT
P+GCA =S+CA	GACTGCGTACA	TGCAG	GCA
P+GCC =S+CC	GACTGCGTACA	TGCAG	GCC
P+GCG =S+CG	GACTGCGTACA	TGCAG	GCG
P+GCT =S+CT	GACTGCGTACA	TGCAG	GCT
P+GGA =S+GA	GACTGCGTACA	TGCAG	GGA
P+GGC =S+GC	GACTGCGTACA	TGCAG	GGC
P+GGG =S+GG	GACTGCGTACA	TGCAG	GGG
P+GGT =S+GT	GACTGCGTACA	TGCAG	GGT
P+GTA =S+TA	GACTGCGTACA	TGCAG	GTA
P+GTC =S+TC	GACTGCGTACA	TGCAG	GTC
P+GTG =S+TG	GACTGCGTACA	TGCAG	GTG
P+GTT =S+TT	GACTGCGTACA	TGCAG	GTT

***Mse*I selective primers** (´5→3´)

Pre-amplification: M+C

Code	CORE	ENZ	EXT
M+CAA	GATGAGTCCTGAG	TAA	CAA
M+CAC	GATGAGTCCTGAG	TAA	CAC
M+CAG	GATGAGTCCTGAG	TAA	CAG
M+CAT	GATGAGTCCTGAG	TAA	CAT
M+CCA	GATGAGTCCTGAG	TAA	CCA
M+CCC	GATGAGTCCTGAG	TAA	CCC
M+CCG	GATGAGTCCTGAG	TAA	CCG
M+CCT	GATGAGTCCTGAG	TAA	CCT
M+CGA	GATGAGTCCTGAG	TAA	CGA
M+CGC	GATGAGTCCTGAG	TAA	CGC
M+CGG	GATGAGTCCTGAG	TAA	CGG
M+CGT	GATGAGTCCTGAG	TAA	CGT
M+CTA	GATGAGTCCTGAG	TAA	CTA
M+CTC	GATGAGTCCTGAG	TAA	CTC
M+CTG	GATGAGTCCTGAG	TAA	CTG
M+CTT	GATGAGTCCTGAG	TAA	CTT

Pre-amplification: M+0

Code	CORE	ENZ	EXT
M+AA	GATGAGTCCTGAG	TAA	AA
M+AC	GATGAGTCCTGAG	TAA	AC
M+AG	GATGAGTCCTGAG	TAA	AG
M+AT	GATGAGTCCTGAG	TAA	AT
M+CA	GATGAGTCCTGAG	TAA	CA
M+CC	GATGAGTCCTGAG	TAA	CC
M+CG	GATGAGTCCTGAG	TAA	CG
M+CT	GATGAGTCCTGAG	TAA	CT
M+GA	GATGAGTCCTGAG	TAA	GA
M+GC	GATGAGTCCTGAG	TAA	GC
M+GG	GATGAGTCCTGAG	TAA	GG
M+GT	GATGAGTCCTGAG	TAA	GT
M+TA	GATGAGTCCTGAG	TAA	TA
M+TC	GATGAGTCCTGAG	TAA	TC
M+TG	GATGAGTCCTGAG	TAA	TG
M+TT	GATGAGTCCTGAG	TAA	TT

The *Eco*RI or *Pst*I selective primers were labeled with fluorescein (Meta-Bion, München) at the 5´ ends. Apart from 3 µl of the diluted pre-selective products and reduction of the reaction volume to 10 µl, the selective amplification assay was the same as that for the pre-selective reaction. After a denature step at 94°C for 2 min, 9 cycles were performed with the following "Touch down" profile: 30 s for denaturing at 94°C, 30 s annealing step (65°C-1°C/cycle), 2 min extension at 72°C. The annealing temperature was subsequently reduced in 9 cycles by 1°C a cycle starting at 65°C in the first cycle and was continued with an annealing temperature at 56°C for the remaining 23 cycles. The profile finished with an additional extension step at 72°C for 2 min. After PCR reaction, products were mixed with formamide dye (98% de-ionized formamide, 10 mM EDTA pH 8.0 and bromo-phenol-blue and xylene-cyanol as tracking dyes) in a one to one ratio and stored at 4°C before loading on the gel. All PCR amplifications were performed in a Gene Amp PCR system 9600 thermocycler (Perkin Elmer Corp., Norwalk).

2.2.2.4 Gel electrophoresis and imaging

First, gel was made with a large glass plate (36 cm x 20 cm), a smaller one (33 cm x 20 cm) and two spacers (0.4 mm). Three sides of the gel plates were sealed with *Tesa* packet sealing tape, leaving the upper part unsealed, and additionally clamps tightened the borders. Because AFLP fingerprinting of potato genomic DNA produces many polymorphism, a 4.5 % polyacrylamide gel was used. The gel matrix was prepared with 40% highly concentrated commercial polyacrylamide finish mixture solution (Acrylamide:Bisacrylamide 19:1, Roth, Karlsruhe), 8 M Urea and 1X TBE buffer. The gel mixture was filtered, stirred and aspirated in a Nalgene Vacuum Filter, pore size 2 µm (Nunc, Wiesbaden) using a running water pump for 15 min at room temperature. Before pouring the gel solution, 260 µl 10% APS (ammonium persulfate) and 60 µl TEMED (N,N,N',N'-tetramethylethylenediamine) were added to 100 ml of gel solution to induce polymerization. After pouring the gel mixture into the prepared gel plates, the blunt side of a comb was inserted into the upper part of the gel and tightened by clamps. After polymerization for at least 1 hour, the *Tesa* sealing tape was removed. Then the gel was pre-heated under denaturing

conditions at constant power (50 W, 1600 V and 250 mA) in 1 X TBE running buffer for a half hour. After short vortex and spin down, selective PCR reactions diluted with formamide dye were denatured at 94°C for 3 min and then quickly chilled on an ice bath for at least 5 min. Denatured sample mixtures (2.5 µl) were loaded and separated for two hours. Then the "Gel-sandwich" was scanned by a laser scanner "Typhoon 9100" (Amersham-Pharmacia, Freiburg) under highly sensitive conditions with an excitation wavelength of 488 nm and a band-pass filter (530 ± 15 nm). The scanned gel image was analyzed with an included software package, FRAGMENT ANALYSIS 1.1 (Amersham-Pharmacia).

2.2.3 Simple Sequenced Repeat (SSR) analysis

Twenty-seven published simple sequenced repeats (SSR)s from Milbourne *et al*. (1998) were used (Table 7). The SSR amplification was performed with 0.1 µM forward primer, 0.1 µM reward primer, 50 ng genomic DNA, 1.5 mM $MgCl_2$, 0.4 mM dNTPs, 0.25 U *Taq* polymerase and 1X reaction buffer in 10 µl total reaction assay using an Eurogentec PCR reaction kit. The annealing temperature of each primer was screened using a "Gradient-Touchdown" PCR profile. Gel concentration electrophoresis was performed as above (2.2.2.4). The gel concentration was varied from 4.5 % to 6 % depending on the prospective fragment size. These SSR primers were not modified at the 5´ ends, so Vistra Green™ (Amersham Pharmacia) was used for imaging bands. It is 10 fold more sensitive than ethidium bromide and permits detection of at least 20 pg/band in both agarose and acrylamide gels. It is thought to bind non-covalently to the DNA backbone. After electrophoresis, one gel glass plate was removed carefully. Vistra Green™ was diluted 1:10,000 in 5 ml of 1X TBE buffer and this dyeing solution was distributed gently onto the gel surface using a small piece of paper. After 5 min of dyeing, the gel was scanned with a wavelength of 488 nm by the laser scanner.

Table 7: List of SSR primers according to Milbourne *et al*. (1998)

Primer	Repeat		Primer sequence 5´- 3´	Product (bp)	Location
STM0001	$(TG)_4(TC)_2(TG)_5$	F	AGTATTCAACCCATTGACTTGGA	170	VI
		R	TAGACAAGCCAAGCTGGAGAA	80-175	
STM0003	$(AC)_9(AT)_9$	F	GGAGAATCATAACAACCAG	141	XII
		R	AATTGTAACTCTGTGTGTGTG		
STM0004	$(AC)_9(AT)_7(AC)_5$	F	CGAGGGCGTAAACTCATGATA	149	VII
		R	AGGTTATTGTGGACACAGTCTTCA		
STM0007	$(AC)_9$	F	GGACAAGCTGTGAAGTTTAT	178	XII
		R	AATTGAGAAAGAGTGTGTGTG		
STM0010	$(TG)_7(TA)_6(TG)_4TA(TG)_5$	F	TCCTTATATGGAGCAAGCA	174	IX
		R	CCAGTAGATAAGTCATCCA		
STM0013	$(AC)_{23}(AT)_6(AG)_9$	F	AACTATCAACTAAATGCCTTTTT	195	V
		R	TTAATATTTTTTACTCGGCTATTG	130	
STM0014	$(GT)_5(AT)_7(GT)_{10}$	F	CAGTCTTCAGCCCATAGG	180	VII,XII
		R	TAAACAATGGTAGACAAGACAAA		
STM0017	$(AC)_3(AT)_2(AC)_4$	F	ATCATGATGACACCTACTATAACC	176	IX
	$(AT)_6(AC)_7$	R	TCCACACCTCTATCTGTTGA		
STM0019	$(AT)_7(GT)_{10}(AT)_4$	F	AATAGGTGTACTGACTCTCAATG	204	VI
	$(GT)_5(GC)_4(GT)_4$	R	TTGAAGTAAAAGTCCTAGTATGTG		
STM0024	$(GTT)_5.(AC)_{12}(AT)_6$	F	CATTACCTTGTGAGATTAGATTG	135	VIII
		R	CATATAAGTAGGAATAGGAGGTTT		
STM0025	$(TG)_{13}$	F	GTTCATGATTGTGAATGCTC	146	XI
		R	ATGACTCAACCCCAAATG		
STM0028	$(AC)_{12}(AT)_5(AG)_8$	F	CATAAATGGTTATACACGCTTTGC	145	VII
		R	TAATGGAGTTCCTGAAAAGAAAGG		
STM0030	compound $((GT+GC)GT)_8$	F	AGAGATCGATGTAAAACACGT	147	XII
		R	GTGGCATTTTGATGGATT		
STM0031	$(AC)_5(AC)_3(GCAC)$	F	CATACGCACGCACGTACAC	172	VII
		R	TTCAACCTATCATTTTGTGAGTCG		
STM0032	$(AC)_7(AC)_5$	F	GGCTGCAGGAATTATGTGTTC	174	XII
		R	GATGTAAAACACGTGTGCGTG	150-175	
STM0037	$(TC)_5(AC)_6.(AC)_7(AT)_4$	F	AATTTAACTTAGAAGATTAGTCTC	90	XI
		R	ATTTGGTTGGGTATGATA	75	
STM0038	$(TA)_4(TG)_{12}$	F	AACTCTAGCAGTATTTGCTTCA	108	II
		R	TTATTTAGCGTCAAATGCATA		
STM0051	$(AC)_5.(AC)_7(AT)_4$	F	TACATACATACACACACGCG	115	X
		R	CTGCAACTTATAGCCTCCA		
STM1003	$(TA)_{10}$	F	CACTTAATATGGAATAGAGGAAGTA	172	VII
		R	GAACTTATGCTTTATCATTCA		
STM1009	$(AT)_{30}$	F	ATTAGCATACGACTCAAC	198	VII,XI
		R	TTATTTTCATTTTTCAGC		
STM1021	$(C)_{17}(CT)_8(AT)_9$	F	GGAGTCAAAGTTTGCTCACATC	181	IX
		R	CACCCTCAACCCCCATATC		
STM1031	$(AT)_{13}$	F	TGTGTTTGTTTTTCTGTAT	276	V
		R	AATTCTATCCTCATCTCTA		
STM1049	$(ATA)_6$	F	CTACCAGTTTGTTGATTGTGGTG	189	I
		R	AGGGACTTTAATTTGTTGGACG		
STM1100	$(TA)_{22}$	F	GCTTCGCCATCTTAATGGTTA	173	VI
		R	TCTTCGAAGGTACGTTTAGACAA		
STM2005	$(CTGTTG)_3$	F	TTTAAGTTCTCAGTTCTGCAGGG	166	XI
		R	GTCATAACCTTTACCATTGCTGGG		
STM2012	$(CA)_3.(GA)_4.(AG)_3.(CGG)_5$	F	GCGGCCGCTTCTCAGCCAA	247	X
		R	TCTCGTTCAATCCACCAGATC		
STM3016	$(GA)_{27}$	F	TCAGAACACCGAATGGAAAAC	151	IV
		R	GCTCCAACTTACTGGTCAAATCC		

2.2.4 Restriction Fragment Length Polymorphism (RFLP) analysis

2.2.4.1 Probes

Restriction Fragment Length Polymorphism (RFLP) analysis was performed for genomic analysis of the dihaploid "Artis" population. The ^{32}P-labelled DNA probes, cDNA of Potato (CP) and Genomic Potato DNA (GP) had been mapped by Gebhardt *et al.*, (1989, 1991) at the Max-Planck-Institute, Köln and were supplied by Dr. Frei (Technische Universität München-Weihenstephan).

2.2.4.2 Restriction enzyme reaction

For probe screening, six randomly selected dihaploid lines were used. Genomic DNA (ca. 5 µg/lane) was digested with 7 different restriction enzymes (Table 8).

Table 8: Cutting sites of the restriction enzymes used in RFLP-analysis

Enzyme/Company	Cutting site		Enzyme/Company	Cutting site	
***Bam*HI**	5´-G↓GATCC-3´	6 bp	***Hind*III**	5´-A↓AGCTT-3´	6 bp
/ Boehringer	3´-CCTAG↑G-5´	/ sticky	/ Boehringer	3´-CCTAG↑G-5´	/ sticky
***Dra*I**	5´-TTT↓AAA-3´	6 bp	***Taq*I**	5´-T↓CGA-3´	4 bp
/ Boehringer	3´-AAA↑TTT-5´	/ blunt	/ Boehringer	3´-AGC↑T-5´	/ sticky
***Eco*RV**	5´-GAT↓ATC-3´	6 bp	***Xba*I**	5´-T↓CTAGA-3´	6 bp
/ Boehringer	3´-CTA↑TAG-5´	/ blunt	/ Boehringer	3´-AGATC↑T-5´	/ sticky

*Eco*RI was already described in Table 3

Three enzyme units per µg DNA, respectively around 15 U per sample, were used in the restriction assay containing the corresponding 1x reaction buffer. The reaction was incubated overnight at 37°C.

2.2.4.3 Gel analysis and blotting

Digested DNA (ca. 5 µg) was separated in a 0.9 % agarose gel in 1X TBE buffer containing 10 µg/ml of ethidium bromide at 30 V, 120 mA overnight. The gel was transferred in 0.25 N HCl solution for 10 min. Then it was denatured in 0.5 M NaOH and 0.5 M NaCl solution for 30 min and neutralized in 0.5 M Tris-HCl (pH 7.5) and 0.5 M NaCl for 30 min. Denatured DNA in the gel was transferred onto a positive charged Nylon membrane (Boehringer, Manheim) by capillary upstream method using alkalic transfer buffer (0.6 M NaCl, 0.4 M NaOH) according to Reed & Mann (1985) overnight. On the following day, the rest salts on the membrane were washed with 2X SSC buffer for 2-3 min. The membranes were dried on Whatmann paper and then baked at 80°C for 2

hours. The baked membranes were stored between papers at room temperature.

2.2.4.4 Probe DNA isolation

Probe DNA was inserted into Bluescript vector (Stratagene). GP probes were released with the restriction enzyme *Pst*I and CP probes with *Eco*RI (Table 9).

Table 9: List of RFLP probes used in this study

Marker	Approximate insert size	Vector	*E.coli* strain	site	Chromosome
CP 13	950 bp	pBluescript	TG-2	*Eco*RI	I
GP 23	1300 bp	pBluescript	TG-2	*Pst*I	II
GP 504	1580 bp	pBluescript	TG-2	*Pst*I	II
GP 26	840 bp	pBluescript	TG-2	*Pst*I	II
GP 303	>500<1000 bp	pBluescript	TG-2	*Pst*I	III
CP 6	480 bp	pBluescript	TG-2	*Eco*RI	III
CP 18	460 bp	pBluescript	TG-2	*Eco*RI	VI
GP 76	900 bp	pBluescript	TG-2	*Pst*I	VI
CP 56	480 bp	pBluescript	TG-2	*Eco*RI	VII
CP 52	420 bp	pBluescript	TG-2	*Eco*RI	VII
GP 68	>500<1000 bp	pBluescript	TG-2	*Pst*I	VIII
GP 24	840 bp	pBluescript	TG-2	*Pst*I	VIII
GP 41	700 bp	pBluescript	TG-2	*Pst*I	IX
CP 51	510 bp	pBluescript	TG-2	*Eco*RI	X, VII
GP 125	1600 bp	pBluescript	TG-2	*Pst*I	XI
CP 57	680 bp	pBluescript	TG-2	*Eco*RI	XI
GP 268	>500<1000 bp	pBluescript	TG-2	*Pst*I	XII
CP 118	470 bp	pBluescript	TG-2	*Eco*RI	XII

Plasmid DNA was isolated by a modified boiling lysis method (Sambrook *et al*., 1989). *E. coli* was cultured in 3 ml LB medium containing 100 mg/l of ampicillin by shaking at 250 rpm in a 37°C incubator overnight. Grown cells were gathered in 1.5 ml tubes by double centrifugation at 1400 rpm (Sigma 1-15, Hamburg) for 30 s. After removing culture medium, the cells were re-suspended with vortex in 400 µl of STET buffer (8% (w/v) Sucrose, 50 mM Tris-HCl (pH 8.0), 50 mM EDTA, 5% (w/v) Triton X-100 containing fresh added 0.2 mg/l lysozyme. For enzymatic lysis, cells were incubated at room temperature for 5 min. DNA and protein of *E. coli* cells were denatured at 99°C in a thermo-mixer (Eppendorf, Hamburg) by shaking with 1400 rpm for 40 s. The mixture was centrifuged at 4°C, 1400 rpm for 10 min. Floated cell debris was removed by hooking with a toothpick and then DNA was precipitated by shaking gently after adding pre-chilled 400 µl of isopropanol and 50 µl of Sodium acetate (pH 4.9) and centrifuged at room temperature with 1400 rpm for 10 min. The pellet was washed with pre-chilled 70 % Ethanol. Remaining Ethanol was removed with a

pipette. The pellet was air-dried for 5 min and suspended in 100 µl of TE buffer containing 10 µg/ml of RNaseA by shaking at 50°C in a thermo-mixer, 1400 rpm for 15 min. One µl of extracted plasmid DNA was used in a 20 µl restriction assay with respectively 3 Units of the appropriate restriction enzymes (*Pst*I, *EcoR*I) and the corresponding 1x reaction buffer. The size of insert was detected after separation in a 1.0 % agarose gel and the target band was cut out under UV light and eluted by JetSorb (GENOMED, Bad Oeynhausen) DNA extraction kit according to manual.

2.2.4.5 Probe DNA labeling

The labeling of the CP- and GP probes was performed using a random hexamer labeling kit (Amersham, Freiburg) according to Feinberg & Vogelstein (1983) with small modifications. 13 µl of ca. 25 ng linear double-stranded probe DNA were first denatured at 95°C for 10 min and then chilled on ice water to stabilize the single-stranded DNA chains. One reaction required 1.5 µl of hexanucleotide random primer mixture, 1.5 µl of dAGT mixture (dATP, dGTP and dTTP), 3.0 µl of [α-^{32}P]-dCTP and 1 µl of Klenow-Fragment (2 U/µl). For random priming, 7 µl of radioactive labeling mixture was added to the single-stranded DNA probe and incubated at 37°C in a thermo-mixer for 1 h with surrounding radioactivity protectors. After incubation the reaction assay was shortly spun down and 10 µl of TE buffer were added. During labeling incubation, Sephadex filters were prepared for purifying the labeled probes. The filter was composed of inserted glass wool at the end of a 1 ml tip that had been autoclaved after coating by pipetting a silicon solution through the tip. Di-sterilized water was dropped into the prepared glass wool filter and then ca. 800 µl of an autoclaved 4 % (w/v) Sephadex solution in TE buffer were added without air bubbles. From the finished probe labeling, 30 µl of the reaction assay were dropped into a Sephadex-filter and subsequently 400 µl of TE buffer was added. The first filtered solution was discarded. With additional 400 µl of TE buffer, the labeled probes were filtered through the Sephadex-filter. The radioactivity of P^{32}-labeled probes was measured and the end volume was calculated in order to reach around $1x10^6$ counts per minutes (cpm) per 1 ml of hybrid DNA solution.

2.2.4.6 Hybridization

The blotted and baked membrane was incubated in pre-heated washing solution I (1X SSC, 0.2 % SDS) at 65°C in a hybridization oven (Bachhofer, Germany) for 30 min. Then the solution was changed to ca. 10 ml of pre-heated hybridization solution (5 X SSC, 0.1 % N-Lauroylsarcosine, 0.02 % SDS, 1 X Blocking solution from Boehringer). After pre-hybridization overnight for new membranes or 2 hours for a stripped membrane, the P^{32}-dCTP labeled DNA probe was denatured at 95°C for 10 min and chilled on an ice bath. Then the calculated volume of DNA probe was added into the hybridization solution with the membrane and incubated for 14-16 hours. The hybridization solution was removed and pre-heated (60°C) washing solution I was added. After incubation for 15 min, the membrane was transferred into a container with new pre-heated washing solution I and further incubated at 60°C for 15 min in a shaking water-bath at 30 rpm. The membrane was rinsed with di-sterilized water and placed on a filter paper for measuring the radioactivity of the membrane. Until reduction to ca. 100 cpm, the membrane was washed with washing solution I and II in a 60°C water bath for ca. 10 min. The membrane was wrapped between plastic foils and then placed into an exposing cassette with X-ray film. This cassette was stored at – 80°C for approximately 3 to 7 days depending on the radioactivity.

2.3 Potato virus Y resistance test

2.3.1 Preliminary examinations

89 *in vitro* lines regenerated from anthers of the cultivar “Assia” were tested by ELISA (Enzyme-Linked Immunosorbent Assay) in order to exclude any virus contamination in the material, especially potato virus Y. Each line was propagated *in vitro* and 20 repeats were pricked out into 96-well trays (Ø 3 cm) and hardened under a net in the greenhouse. Additionally, *in vitro* plants of the cultivar “Assia” and “Walli” were prepared as negative and positive controls. After 2 weeks, these lines were planted into pots (Ø 9 cm) in May 2000.

2.3.2 Infection tests

2.3.2.1 Primary infections

Potato virus Y inoculation was performed after three weeks using mechanical infection methods from the potato virus test department of the Bayerische Landesanstalt für Landwirtschaft. For PVY infection, 4 different strains (PVY^{o}, PVY^{c}, PVY^{N} and PVY^{NTN}) were inoculated together. Two weeks after PVY inoculation, symptoms from primary infection were observed in several plants. Mini-tubers were harvested 4 months after infection.

2.3.2.2 Analysis of secondary infections

Harvested mini-tubers were stored for ca. 6 weeks and treated by Rindite for artificial breaking of tuber dormancy. Twenty tubers per line were planted into pots in the greenhouse on December 2000. After three weeks, when the plants had grown 15 to 20 cm, a few leaves of each pot were analyzed by ELISA. Symptoms in the leaves were evaluated in the shade and noted as mosaic or wrinkle symptoms.

2.3.3 Selection of resistant and susceptible lines

Based on the ELISA results and symptom evaluation, the Assia population was divided into pools with resistant or susceptible individuals. For Bulked Segregant Analysis, a small number of resistant and susceptible lines with unequivocal test results in all repeats were selected.

2.4 *Ry_{sto}* marker development and mapping

2.4.1 *Ry_{adg}* marker application

In order to test the applicability of *Ry_{adg}* SCAR markers (Table 10) from Kasai *et al*. (2000), ten German cultivars (“Bettina”, “Forelle”, “Kuras”, “Laura”, “Petra”, “Artis”, “Sibu”, “Tomba”, “Ute” and “Sempra”) with good resistance data to PVY based on data of the Beschreibende Sortenliste were analyzed. Cultivar “Assia” was used as a control.

Table 10: Ry_{adg} SCAR marker primer list

Primer	Sequence
3.3.3as	5´-ACTTAACTGCATCATGTTCAAG-3´
3.3.3s	5´-ATACACTCATCTAAATTTGATGG-3´
ADG21 F	5´-AGTTCTAGTTGTGCTTGATAAC-3´
ADG21 R	5´-GTTATCAAGCACAACTAGAACT-3´
ADG22 R	5´-CAAGATCCCCTGCTAGGTATTT-3´
ADG23 R	5´-AGGATATACGGCATCATTTTTCCGA-3´

2.4.2 Bulked Segregant Analysis

Based on the results of PVY-ELISA test and symptom observation, 2 pools composed of 12 resistant lines (R pool) and 12 susceptible lines (S pool) were selected. In each pool, DNA of 8 lines was mixed and screened together with unmixed DNA of 4 lines by 480 different selective primer combinations of E+A/M+C and P+G/M+C in AFLP analysis. 27 selected AFLP primer combinations (Table 11) were tested again with non-mixed DNA of each pool and in addition with DNA of 10 German potato cultivars according to 2.4.1.

Table 11: Selected primer combinations after Bulked Segregant Analysis

***Pst*I/*Mse*I assay**		***EcoR*I/*Mse*I assay**	
P+GAC/M+CAT	P+GGA/M+CTC	E+AAA/M+CTG	E+ATC/M+CGC
P+GAG/M+CAT	P+GGC/M+CAT	E+AAC/M+CAC	E+ATT/M+CGC
P+GAG/M+CGT	P+GGC/M+CGA	E+ACC/M+CAG	E+AGT/M+CAT
P+GCA/M+CAG	P+GGT/M+CAC	E+ACC/M+CGC	E+AGT/M+CCT
P+GCA/M+CGT	P+GGT/M+CAT	E+ACC/M+CTC	E+AGT/M+CTA
P+GCC/M+CAT	P+GTA/M+CGG	E+ACT/M+CGA	
P+GCT/M+CCA	P+GTT/M+CAT	E+ACT/M+CGG	
P+GGA/M+CGA			

2.4.3 Mapping of Ry_{sto}

2.4.3.1 Polymorphism analysis

From the “Assia” population, 57 clearly different individuals were analyzed with 14 selected primer combinations from Bulked Segregant Analysis in E+A/M+C and P+G/M+C assays and 19 other selective amplification primer combinations showing clear polymorphism (Table 12). From these 33 primer combinations, the bands showing clear polymorphism were scored using the program AFLP-Quantar™ 1.0 (Keygene). In order to find the chromosome localization of Ry_{sto}, STM primers according to 2.2.3 and additionally, the published PCR primer pair

GP268 (Bryan *et al.*, 2002) with the sequence 5´-AACCCGAAGTAACGCCTAAG-3´, 5´-CCATTACCCCAAATCAACAT-3´ was tested in our population.

Table 12: Selected primer combinations for the Ry_{sto} linkage analysis

***Pst*I/*Mse*I assay**		***EcoR*I/*Mse*I assay**	
P+GAC/M+CAT	P+GCA/M+CGT	E+AAC/M+CAC	E+AAT/M+CTC
P+GAG/M+CAT	P+GCC/M+CAG	E+ACC/M+CGC	E+ACC/M+CAC
P+GAG/M+CGT	P+GCG/M+CAA	E+ACC/M+CTC	E+ACG/M+CCT
P+GCC/M+CAT	P+GCT/M+CGA	E+ACT/M+CGA	E+AGA/M+CAT
P+GGA/M+CGA	P+GGT/M+CTT	E+ACT/M+CGG	E+AGC/M+CAG
P+GGA/M+CTC	P+GTA/M+CAA	E+AGT/M+CAT	E+AGT/M+CCC
P+GGC/M+CGA	P+GTC/M+CTA	E+ATC/M+CGC	E+ATA/M+CCT
P+GAT/M+CGG	P+GTT/M+CAG	E+ACA/M+CAC	E+ATG/M+CCT
		E+AAG/M+CGA	

2.4.3.2 Linkage grouping

According to resistance segregation data in the “Assia” population, it can be inferred that “Assia” has the dominant PVY resistance gene in simplex constitution: *Ryryryry.* All scored AFLP polymorphism markers and anchor markers were tested by a X^2 test at the significance level of p=0.05 for one to one segregation. The program “Mapmaker” (version 3.0) was used for linkage analysis with these segregating markers converted to a F_2 backcross population marker code under Kosambi function using a LOD (Logarithm of odds) threshold of 5.0 and maximum distance of 25 cM.

2.4.4 Cloning of Ry_{sto} markers

One AFLP-Ry_{sto} marker fragment (P+GAG/M+CAT) was cut out from a denatured 5 % poly-acrylamide gel and put into a cup containing 100 µl of di-sterilized water. The cup was incubated at room temperature for 2 min. After vortex, it was centrifuged at 5000 rpm (Sigma 1-15) for 1 min and 50 µl of the supernatant were transferred into a new tube. With selective primers, 1 µl of the elution product was used for re-amplification under pre-selective amplification conditions. Re-amplified products were analyzed in a 2 % agarose gel. The target fragment was eluted out of the corresponding agarose gel using a JetSorb extraction kit (GENOMED). The concentration of the eluted products was measured with a spectrophotometer (Amersham-Pharmacia). Using a TOPO TA cloning® kit (Invitrogen, Karlsruhe), the fragment was cloned

according to the instruction manual. At the following day, 24 colonies were picked and incubated in 3 ml of LB medium for plamid isolation. The cloned plasmids were isolated with boiling preparation (Sambrook *et al*., 1989) and the inserted DNA fragments were detected after cutting with restriction enzyme *Eco*RI at the poly-linker site of the vector and analyzed in a 1.5% agarose gel. Selected plasmids were purified by Jetquick Plasmid Miniprep Spin kit (GENOMED) and sequenced by the company Medigenomix (Martinsried, Germany). The sequence data were analyzed by the sequence analysis program Vector NTI ™ version 6.0 (InforMax®, Invitrogen life technologies).

2.4.5 Development of a PCR marker based selection system

One Ry_{sto} PCR marker candidate was designed in alignment with sequence information under consideration of a GC content over 40 % and an annealing temperature of around 60°C in the forward and reward primer sequence. It was first tested in the pools of the dihaploid "Assia" population that were used for Bulked Segregant Analysis and furthermore in all 57 lines of the dihaploid "Assia" population. In addition, the Ry_{sto} PCR primer candidate was tested in resistant and susceptible pools of 110 cultivated potatoes based on selection data from AFLP results.

2.4.6 Pedigree analysis

The genetic background of the cultivars selected by Ry_{sto} markers for extreme resistance to PVY was analyzed using a Potato Pedigree Database from Wageningen (The Netherlands), http://www.dpw.wau.nl/pv/. For some Polish cultivars, pedigree data were received from Prof. Zimnoch-Guzowska at the Plant Breeding and Acclimatization Institute Radzików in Poland. The data for the cultivars "Tomba" and "Oktan" were received from Dr. Hofferbert, (Böhm-Nordkartoffel Agrarproduktion, Zuchtstation Ebstorf) and for "Jumbo", "Assia", "Arosa" and "Alwara" from the potato breeding department of the Bayerische Landesanstalt für Landwirtschaft (LfL), Freising.

2.5 Chip color quality after long term storage at 4°C

2.5.1 Evaluation of chip quality and related trials

2.5.1.1 Field trials and storage

From 1999 to 2001, the dihaploid and tetraploid Artis populations were tested in the field. In 1999, two tubers per line were planted at the location “Schlüter”. In 2000 five tubers per line were planted at “Pulling” with two repeats. In 2001 three tubers per line were planted at both locations Schlüter and Pulling with three randomized repeats per location (Table 13).

Table 13: Data for field trials and chip quality evaluation

		Field							
Year	Popul-ation	Location	repeats	Order	Nr. of tubers per row	Planting	Harvest	Storage period	Chip test
1999	2x	Schlüter	1	Following Nr.	2	April 99	Oct. 99	From 02.10.99 to 03. 04. 00	04.04.00
	4x	Schlüter	1	Following Nr.	2	April 99	Oct. 99		04.04.00
2000	2x	Pulling	2	Following Nr.	5	26.04.00	04.10.00	From 20.10.00 to 09.04.01	10.04.01
	4x	Pulling	2	Following Nr.	5	26.04.00	05.10.00		10.04.01
2001	2x	Schlüter	3	Randomized	3	27.04.01	13.09.01	From 20.09.01 to 19-21.02	20-22.02.02
	2x	Pulling	3	Randomized	3	27.04.01	14.09.01	From 20.09.01 to 06.03.02	07.03.02
	4x	Schlüter	3	Randomized	3	27.04.01	11.09.01	From 20.09.01 to 12-13.04.02	13-14.02.02
	4x	Pulling	3	Randomized	3	27.04.01	14.09.01	From 20.09.01 to 05.03.02	06.03.02

After harvest, 10 tubers of each line were stored at 4°C in a dark air-conditioned room until one day before chip frying. As control cultivars, every year “Artis”, “Sempra”, “Tessi” and “Panda” were planted at every location with at least three repeats per location.

2.5.1.2 Determination of chip quality

After long term storage (5 to 7 months) at 4°C, ten tubers per line were reconditioned overnight (ca. 18 h) to room temperature. Then the tubers were washed and peeled by a peeling machine (Flott 25K, Frankfurt am Main) and cut into slices of 1.2 mm thickness by a potato slice-cutting machine (Flott S, Frankfurt am Main). The tuber slices were put into a net bag and washed in running water to remove free starch rests. Rest water was removed by a dehydrator machine (Thomas, Germany). The chip was fried at 160°C in oil for

3 min in a frying machine (Roede, Germany) and fresh fried chip were dried on a kitchen paper towel to remove remaining oil. Within 2 h after frying, the chip color was scored with notes 1 to 9 (dark to light). The phenotypic evaluation data resulted from two independent testers who made chip color comparisons to the standard cultivars “Artis”, “Sempra” and “Tessi”. The chip test results in 2001 were additionally documented by a digital camera under constant illumination conditions together with calibrated Color cards developed by the Institute for Storage and Processing of agricultural Produce (Wageningen, The Netherlands) for the European Association for Potato Research for quality evaluation of potato chip. The results of chip color evaluation were compared with the photographic evaluation.

2.5.1.3 Measurement of reducing sugar content

After long term cold storage, one day before frying chip, one tuber of every line was randomly selected and cored in the middle of the tuber using a cork borer (∅ 2.0 cm). From this probe, the middle part (ca. 3 cm) of bored tuber tissue was cut and completely dried in a vacuum freeze-drier (UniEquip, Martinsried). Dried potato samples were finely grained using a ball-miller machine (Retsch, Haan) with 200 rpm for 2 min. The powder was conserved in a plastic zipper bag (6 cm x 8 cm) for preventing the absorption of humidity. The method for extraction of reducing sugars was obtained from Dr. Gebhardt from the Max Planck Institute, Cologne. From the dried potato powder, 100 mg of each line were transferred into a 1.5 ml micro-screw tube having an O-ring in the lid (Sarstedt, Nümbrecht). Then 1 ml of ethanol (80 % [v/v]) was added to the probe. The probes were immediately closed and mixed by vortex. In order to increase the efficiency of re-suspension, the tubes were slanted and incubated in a water bath (80°C) by maximum shaking for 10 min. These tubes were transferred to a preheated thermo-mixer and incubated at 80°C with shaking (1400 rpm) for 1 h. After centrifugation (Sigma 1-15) with 1400 rpm for 10 min, 800 µl of the supernatant were transferred into a 1.5 ml tube and incompletely evaporated by a SpeedVac (Jouan, Unterhaching) with a running water pump. The final volume was adjusted to 1000 µl with di-sterilized water and the probes were stored at –20°C. The measurement of glucose and fructose was performed as soon as possible after extraction due to possible bacterial growth

in the extracted solution. The content of the extracted reducing sugars was analyzed by an enzymatic reaction UV method kit (R-Biopharm AG, Darmstadt). The reaction volume was reduced 10 fold and measurement was performed according to manual with a Micro-plate-reader containing a 340 nm filter at 25°C and the program SOFTmax® Pro version 3.1 (Molecular Devices, Ismaning). The resulting raw data were re-calculated by the following formula as suggested in the manual:

$$C = \frac{V \times MW}{\varepsilon \times d \times v \times 1000} \times \Delta A \text{ [g/l]}$$

V = Final volume (ml)

v = Sample volume (ml)

MW = Molecular weight of the substance to be assayed (g/mol)

D = Light path (cm) (1 in this measurement)

ε = Extinction coefficient of NADPH at: 340 nm = 6.3 ($l \times mmol^{-1} \times cm^{-1}$)

F = Dilution factor (10 in this measurement)

ΔA = Difference of absorbance value in spectrophotometer according to enzyme reaction

For D-glucose:

$$C = \frac{3.020 \times 180.16}{6.3 \times 1.00 \times 0.100 \times 1000} \times \Delta A_{D\text{-glucose}} \text{ [g D-glucose / sample solution]}$$

For D-fructose:

$$C = \frac{3.040 \times 180.16}{6.3 \times 1.00 \times 0.100 \times 1000} \times \Delta A_{D\text{-fructose}} \text{ [g D-fructose / sample solution]}$$

2.5.1.4 Determination of starch content

In 2001, a few days after harvest, tuber starch content of 83 dihaploid and 101 tetraploid lines was determined by measuring the specific gravity (weight in air/weight in air - weight in water) based on the method of Von Scheele *et al.* (1937) using a starch balance (Satorius, Göttingen). The calculation formula is as follows:

$$\% \text{ Starch} = 17.546 + 199.07 \times (\text{specific gravity} - 1.0988).$$

After cold storage, the starch content of the same samples was measured again one day before chipping. After measurement of starch content, the samples were reconditioned at room temperature for ca. 12 to 18 h.

2.6 Construction of genetic map for a good chip color quality after long term storage at 4°C

2.6.1 Molecular marker analysis

2.6.1.1 Genetic analysis

For characterization of the dihaploid "Artis" population, 108 selective AFLP primer combinations were used with two different restriction enzyme combination systems: 89 selective primer combinations of E+A/M+C pre-amplification and 19 selective primer amplifications of P+G/M+C pre-amplification (Table 14).

Table 14: 108 selected primer combinations for constructing linkage groups in the dihaploid "Artis" population

***Pst*I/*Mse*I assay**	***EcoR*I/*Mse*I assay**		
P+GAG/M+CGT	E+AAA/M+CCA	E+AAT/M+CCC	E+ATC/M+CCT
P+GCC/M+CCT	E+AAA/M+CCC	E+AAT/M+CCT	E+ATC/M+CGA
P+GCT/M+CAT	E+AAA/M+CCT	E+AAT/M+CGA	E+ATC/M+CGC
P+GGT/M+CGG	E+AAA/M+CGC	E+AAT/M+CGC	E+ATC/M+CGG
P+GTT/M+CAG	E+AAC/M+CAA	E+AAT/M+CGG	E+ATC/M+CGT
P+GAA/M+CGA*	E+AAC/M+CAC	E+AAT/M+CGT	E+ATC/M+CTC
P+GAC/M+CAT*	E+AAC/M+CAG	E+AAT/M+CTA	E+ATC/M+CTG
P+GAG/M+CAT*	E+AAC/M+CAT	E+AAT/M+CTC	E+ATC/M+CTT
P+GCG/M+CAA*	E+AAC/M+CCA	E+AAT/M+CTG	E+ATG/M+CAC
P+GCT/M+CTT*	E+AAC/M+CCC	E+ACA/M+CAC	E+ATG/M+CCC
P+GGA/M+CTT*	E+AAC/M+CCG	E+ACA/M+CAG	E+ATG/M+CCG
P+GGC/M+CTA*	E+AAC/M+CGA	E+ACA/M+CAT	E+ATG/M+CCT
P+GGC/M+CTC*	E+AAC/M+CGC	E+ACA/M+CCG	E+ATG/M+CGT
P+GGG/M+CAC*	E+AAC/M+CGG	E+ACA/M+CCT	E+ATG/M+CTA
P+GGT/M+CTT*	E+AAC/M+CGT	E+ACA/M+CGA	E+ATG/M+CTC
P+GTA/M+CTG*	E+AAC/M+CTA	E+ACA/M+CGG	E+ATT/M+CAC
P+GTC/M+CTT*	E+AAC/M+CTC	E+ACA/M+CGT	E+ATT/M+CAT
P+GTG/M+CGG*	E+AAG/M+CAC	E+ACA/M+CTA	E+ATT/M+CCA
P+GTT/M+CTG	E+AAG/M+CAG	E+ACA/M+CTC	E+ATT/M+CCC
	E+AAG/M+CCA	E+ACA/M+CTG	E+ATT/M+CCG
	E+AAG/M+CCC	E+ACC/M+CAC	E+ATT/M+CCT
	E+AAG/M+CCG	E+ACC/M+CAG	E+ATT/M+CGA
	E+AAG/M+CGA	E+ACC/M+CAT	E+ATT/M+CGC
	E+AAG/M+CGC	E+ACC/M+CCA	E+ATT/M+CGG
	E+AAG/M+CGG	E+ACC/M+CCC	E+ATT/M+CGT
	E+AAG/M+CGT	E+ATC/M+CAG	E+ATT/M+CTA
	E+AAG/M+CTC	E+ATC/M+CAT	E+ATT/M+CTC
	E+AAG/M+CTG	E+ATC/M+CCA	E+ATT/M+CTG
	E+AAT/M+CAG	E+ATC/M+CCC	E+ATT/M+CTT
	E+AAT/M+CCA	E+ATC/M+CCG	

* indicated primer combinations were used for analysis of the tetraploid "Artis" population

As anchor markers, 27 SSR markers (Table 7) and 18 RFLP probes (Table 9) were used. Thirteen randomly selected AFLP primer combinations out of the 108 selective amplification primers from the dihaploid population analysis were used to verify the results in the tetraploid “Artis” population. The selected primer combinations were marked by asterisk in table 14.

2.6.1.2 Segregation analysis

Clear polymorphic bands were scored out on presence and absence using the program AFLP-Quantar™ 1.0 (Keygene) and personal verification. The marker segregation data was tested for segregation distortion with an X^2 test for goodness of fit to appropriate expected segregation ratios with a significance level of p=0.05:

$$X^2 = \frac{(X_{A\ \text{observation}} - E_{A\ \text{expectation}})^2}{E_{A\ \text{expectation}}} + \frac{(X_{a\ \text{observation}} - E_{a\ \text{expectation}})^2}{E_{a\ \text{expectation}}}$$

The value of the X^2 test must be below 3.847 (Weber, 1978). The expected segregating ratios in the dihaploid population are for simplex 1 to 1 (*Aa:aa*), and for duplex 5:1 (*A-:aa*). In the tetraploid population, for simplex a 1 to 1 (*Aaaa:aaaa*) and for duplex a 5:1 (*A-aa:aaaa*) segregation was expected if the marker dosage was derived only from the paternal or maternal parent. If the alleles were from both parents, segregation in the ratio 3 to 1 (*A-aa:aaaa*) or the ratio 11 to 1 (*A-aa:aaaa*) could occur.

2.6.1.3 Linkage group analysis

Genotypic similarity was tested by JoinMap® 3.0 (Plant Research International VB, Wageningen) before linkage analysis. Simplex segregating alleles from the dihaploid population were analyzed with a F_1 backcross population model using a LOD threshold of 5.0 and a maximum distance of 25 cM with the mapping function of Kosambi for constructing linkage groups. Some linkage groups were detected by the alignment of AFLP markers with the Ultra High Density (UHD) map database from http://potatodbase.dpw.wau.nl/.

2.7 QTL-Analysis for good chip color quality after long term storage at 4°C

2.7.1 Data analysis

2.7.1.1 Normality test

Normal distribution of each of the agronomic trait mean values was tested by the "PROC UNIVARIATE" program in SAS (release 8.2) based on the null hypothesis that the input data values are a random sample from a normal distribution. The Shapiro-Wilk statistic (W) is included in the normality test of the "PROC UNIVARIATE" program. The value of W must be greater than zero and less than or equal to one. Small values of W lead to rejection of the null hypothesis of normality.

2.7.1.2 Correlation analysis

Pearson correlation analysis was used between agronomic traits in SAS (release 8.2). One-way ANOVA (Analysis of variance) is an analysis technique used for experimental data in which there is a continuous response variable and a single independent classification variable.

2.7.1.3 Simple marker regression analysis

Generalized Linear models (GLM) were used to analyze the genetic marker data in comparison to good chip quality, reducing sugar content and starch content by the statistical computer program SAS (release 8.2). Regression analysis was performed for evaluation between selected markers through GLM and phenotypic data using "PROC REG" program and a stepwise selection option. The stepwise selection process terminates if no further variable can be added to the model or if the variable just entered into the model is the only variable removed in the subsequent backward elimination (SAS User´s guide, 2000).

2.7.1.4 Analysis of QTL positions on the genetic map

MapQTL® version 4.0 (DLO Wageningen, UR-Centre for Biometry) was used to calculate QTL positions on genetic maps with the mean value of chip color. First, the Kruskal-Wallis test was performed with all molecular markers including

un-linked molecular markers for good chip quality, reducing sugar content after storage, and starch content data. The Kruskal-Wallis statistic can be applied when a locus segregates into only two genotype classes, such as in a backcross (*A:a*), which was used in this study according to the MapQTL manual. Interval mapping was analyzed with at least 1 cM interval, and further multiple QTL model (MQM) mapping was performed according to the MapQTL program and manual.

3 Results

3.1 Extreme resistance to PVY (*Ry$_{sto}$*)

3.1.1 *Ry$_{adg}$* marker application

Ten potato cultivars were analyzed with published *Ry$_{adg}$* SCAR markers (Kasai *et al.*, 2000). The amplified polymorphism of published *Ry$_{adg}$* SCAR markers did not coincide with the resistance evaluation data of ten selected cultivars. The PCR products from primer 3.3.3s and ADG 23 are presented in Fig. 4. Unfortunately, these *Ry$_{adg}$* markers were not suitable to select resistance derived from *S. stoloniferum*.

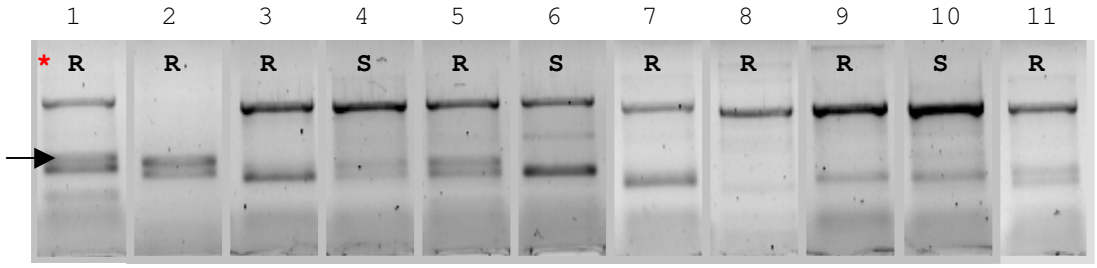


Fig. 4: Analysis of 10 potato cultivars with *Ry$_{adg}$* SCAR markers (3.3.3s and ADG23) Lane 1 “Bettina”, Lane 2 “Forelle”, Lane 3 “Kuras”, Lane 4 “Laura”, Lane 5 “Petra”, Lane 6 “Artis”, Lane 7 “Sibu”, Lane 8 “Tomba”, Lane 9 “Ute”, Lane 10 “Sempra”, Lane 11 “Assia” (Control) * Resistance evaluation data of cultivars according to Bundessortenamt

3.1.2 Evaluation of PVY resistance in a segregating dihaploid population

3.1.2.1 Analysis of primary infections

PVY could not be detected in any *in vitro* material. For the PVY infection test, 89 lines derived from “Assia” by anther culture were used with 20 repeats per line. Among the 89 lines, 19 lines were determined to be mixoploid by flow cytometry and phenotypic segregation. Two weeks after mechanical infection of the seedlings in the greenhouse with PVY, phenotypic response was clearly visible (Fig. 5) mainly in form of mosaic symptoms. However, many of the seedlings were too small to select the resistant lines exactly.

Fig. 5: Phenotypic response to PVY two weeks after infection

3.1.2.2 Analysis of secondary infections

The phenotypic symptoms caused by PVY could be most clearly seen in plants derived from tubers rather than those derived from the infected seedlings because of more vigorous growth (Fig. 6). Also PVY infection was already manifested in the harvested tubers. In some plants necrosis could be observed.

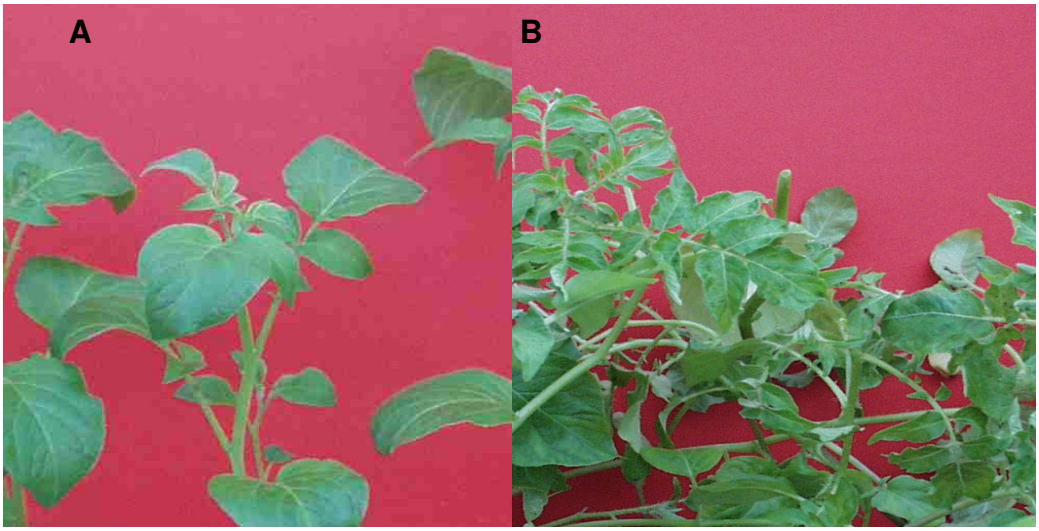


Fig. 6: Phenotypic evaluation of secondary infections of PVY
A: resistant B: susceptible plants

For mapping, 51 exactly dihaploid lines were intensively observed and analyzed by ELISA (Table 15). No phenotypic results could be obtained for 6 lines (P 22, P37, P 38, P 70, P 100 and P 101) because the tubers were too small and did not sprout.

Table 15: Results of the potato virus Y infection test for 51 exact dihaploid lines

Line	[1]Resistance level by ELISA	[2]Phenotypic evaluation	[3]Pool R	S	Line	[1]Resistance level by ELISA	[2]Phenotypic evaluation	[3]Pool R	S
P 1	42 %	S		X	P 69	0 %	R	X	
P 2	25 %	S			P 72	0 %	S		
P 3	5.26 %	S			P 81	0 %	R	X	
P 3.1	12.5 %	S			P 85	21 %	S		X
P 4	15.7 %	S			P 86	0 %	R	X	
P 5	0 %	R			P 87	50 %	S		X
P 9.1.1	42.1 %	S		X	P 98	0 %	R	X	
P 11	0 %	R			P 99	0 %	R		
P 12.2	52.9 %	S		X	P 102	0 %	R	X	
P 15	0 %	R	X		P 103	30 %	S		X
P 19	47.3 %	S		X	P 104	35 %	S		
P 26	50 %	S			P 109	52.9 %	S		
P 27	0 %	R	X		P 110	0 %	R		
P 30	27.3 %	S			P 113	0 %	R		
P 33	0 %	R			P 115	0 %	R		
P 34	0 %	R			P 118	15.7 %	S		
P 35	77.7 %	S		X	P 120	0 %	R	X	
P 36	25 %	S			P 123	15 %	S		
P 39	0 %	R	X		P 125	0 %	R		
P 41	0 %	R	X		P 63a	0 %	R	X	
P 49	0 %	R	X		P 76a	100 %	S		X
P 50	5.5 %	S			P 76b	33.3 %	S		X
P 51	16.6 %	S			P 76c	0 %	S		
P 60	37.5 %	S		X	P 76d	0 %	R		
P 61	0 %	R			P 78a	25 %	S		X
P 66	42.8 %	S							

[1] indicating percentage of positive reaction to PVY by ELISA from 20 times repeats (0% resistant, 100% high susceptible)
[2] R stands for resistance and S for susceptibility
[3] "X" indicates selection for composing R and S pools for Bulked Segregant Analysis.

Results of ELISA and phenotypic symptom evaluation were compared and resistant and susceptible lines were selected for composing pools for further Bulked Segregant Analysis. The basic selection of resistant lines was performed very rigorously. First, resistant lines had to be completely free of PVY in ELISA in all repeats. PVY was not detected in these plants by ELISA. In P 72 and P 76c, the virus was not detected by ELISA but very light mild mosaic symptoms were observed. Therefore, as second condition no mosaic symptoms, wrinkle or necrosis has to be visible by naked eye evaluation. Considering these items, the 12 lines selected for the resistant pool were P 15, P 27, P 39, P 41, P 49, P 69, P 81, P 86, P 98, P 102, P 120 and P 63a, while the susceptible pool consisted of the 12 lines P 1, P 9.1.1, P 12.2, P 19, P 35, P 60, P 85, P 87, P 103, P 76a, P 76b and P 78a. In table 15, these 24 lines are marked by "X". The results show a phenotypic segregation of 23 resistant and 28 susceptible lines. The genetic constitution of extreme resistance to PVY of cv. "Assia" could be assumed as simplex (*Ryryryry*), which results in a nearly 1:1 segregation ratio (*Ryry*:*ryry*) in the primary dihaploid population.

3.1.3 Bulked Segregant Analysis

DNA of 12 resistant lines (R-pool) and 12 susceptible lines (S-pool) was analyzed with 480 selective primer combinations in Bulked Segregant Analysis. Fifteen Ry_{sto} AFLP marker candidates out of the *Pst*I/*Mse*I assay and 12 Ry_{sto} AFLP marker candidates out of the *Eco*RI/*Mse*I assay could be selected. These marker candidates were tested again with non-mixed DNA from each line of the R and S pool. Seven markers from the *Pst*I/*Mse*I assay and 7 markers from the *Eco*RI/*Mse*I assay differentiated clearly resistant and susceptible lines (Fig. 7): P+GAC/M+CAT, P+GAG/M+CAT, P+GAG/M+CGT, P+GCC/M+CAT, P+GGA/M+CGA, P+GGA/M+CTC, P+GGC/M+CGA, E+AAC/M+CAC, E+ACC/M+CGC, E+ACC/M+CTC, E+ACT/M+CGA, E+ACT/M+CGG, E+AGT/M+CAT, E+ATC/M+CGC.

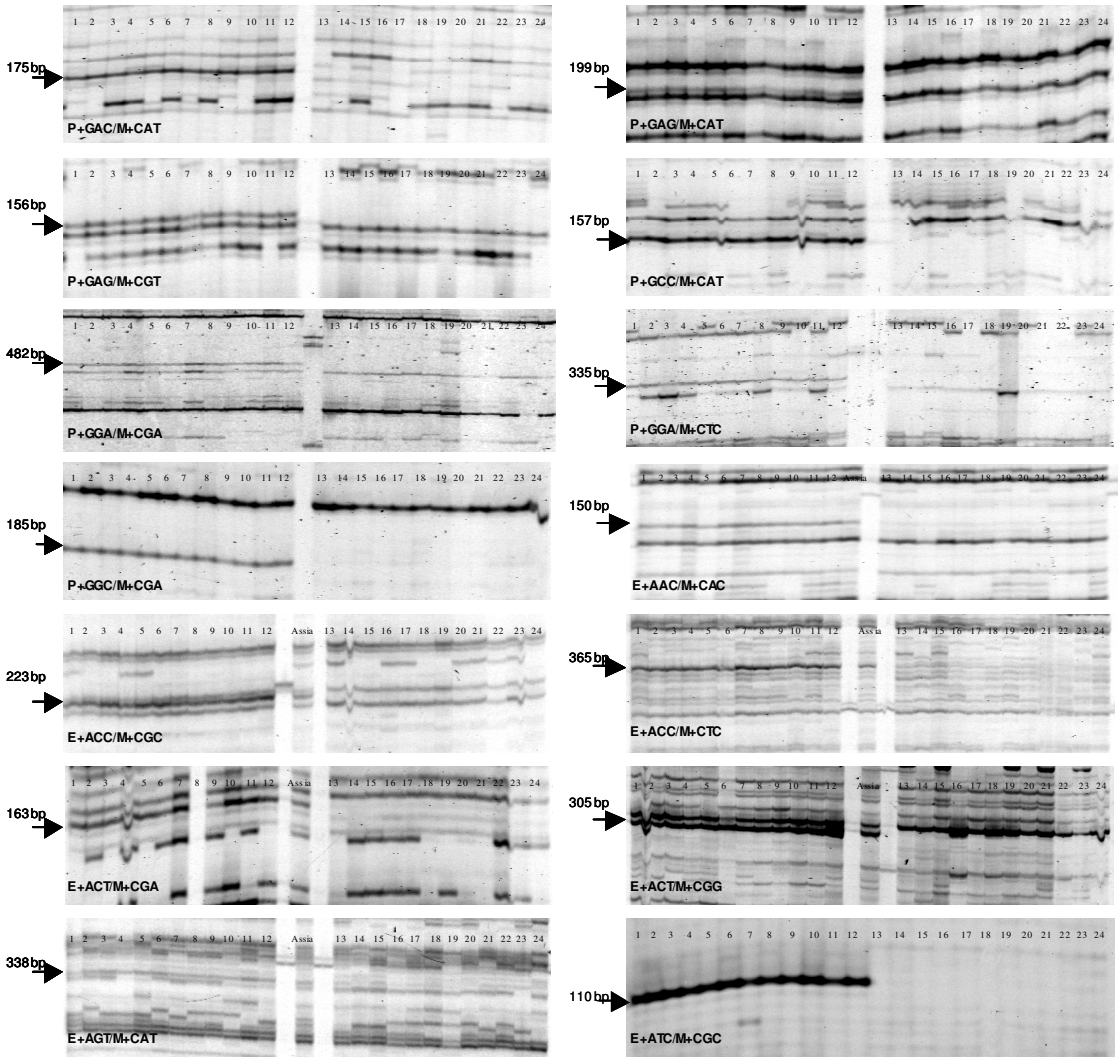


Fig. 7: Evaluation of 14 Ry_{sto} AFLP marker candidates with 24 dihaploid "Assia" lines
Lane 1 to 12 resistant, lane 13 to 24 susceptible lines

3.1.4 Verification of marker candidates

As a control for marker selection, the selected 27 *Ry$_{sto}$* AFLP marker candidates from Bulked Segregant Analysis were tested simultaneously in 10 potato cultivars having good reported data for PVY-resistance by the Bundessortenamt. Based on phenotype data and experience from our institute, cultivars “Bettina”, “Forelle”, “Kuras”, “Petra”, “Sibu”, “Tomba” and “Ute” were expected to show extreme resistance whereas cultivars “Laura”, “Artis” and “Sempra” were expected to be susceptible. According to lane orders, a polymorphism profile of **11101011101** (1=resistant, 0=susceptible) should be visible. The results of this evaluation (Fig. 8) coincided with the results from 3.1.2 except for one marker (E+ACT/M+CGA).

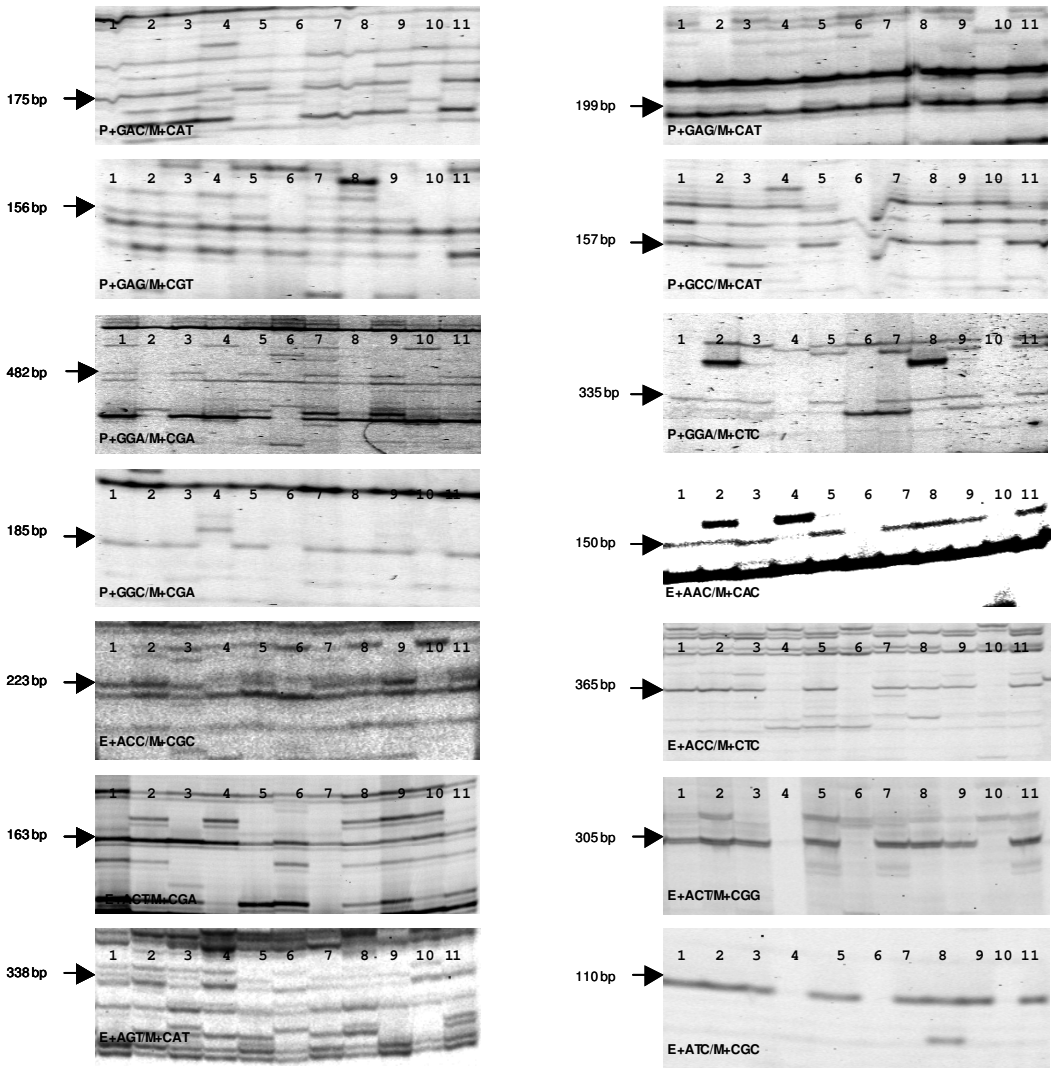


Fig. 8: Evaluation of 14 *Ry$_{sto}$* AFLP marker candidates with 10 potato cultivars
Lane 1 “Bettina”, lane 2 “Forelle”, lane 3 “Kuras”, lane 4 “Laura”, lane 5 “Petra”, lane 6 “Artis”, lane 7 “Sibu”, lane 8 “Tomba”, lane 9 “Ute”, lane 10 “Sempra”, lane 11 “Assia” (control)

3.1.5 Mapping of *Ry$_{sto}$*

For localization of the *Ry$_{sto}$* markers, a total of 33 selective amplification primer combinations were applied in AFLP analysis to 70 lines of the dihaploid "Assia" population. Through AFLP assay, 13 lines were detected as homologous copies of correspondent lines. These 13 copies originated from the same anther. However, in the case of lines P 76a, P 76b, P 76c and P 76d, also derived from the same anther, P 76a P 76b and P 76c were found to be susceptible to PVY, whereas P 76d was resistant. The phenotypic data of 57 lines were compared to genotypic 0/1 matrix data of selected markers. Using a X^2 test with a significance level of $p < 0.05$, 306 one to one segregating AFLP markers were selected from a total of 620 polymorphic bands produced with 33 selective amplification primer combinations. Recombination was not found in *Ry$_{sto}$* markers analysis of the six lines P 22, P37, P 38, P 70, P 100 and P 101 without phenotype results. The markers declared 5 lines as resistant to PVY, except for P22.

Since *Ry$_{sto}$* was reported on chromosome XI (Brigneti *et al.*, 1997), the chromosome XI linkage was tried with 306 simplex AFLP markers and SSR markers anchoring to chromosome XI, but the linkage failed to land the *Ry$_{sto}$* onto chromosome XI and the *Ry$_{sto}$* markers were just linked together. Consequently, *Ry$_{sto}$* could not be localized on chromosome XI. Further SSR markers were tested in order to find the right *Ry$_{sto}$* localization in the dihaploid "Assia" population. One band of the SSR marker STM0003 with a size of 111 bp was co-segregated with three AFLP markers and the phenotypic data of 57 individuals of the population. The *Ry$_{sto\ gene}$* was therefore detected to be linked to chromosome XII (Fig. 9). The comparison with other linkage maps showed that STM0003 is closely linked to GP268 (Milbourne *et al.*, 1998). However, using published GP268 primers according to Bryan *et al.* (2002), no polymorphic band could be detected in our population.

From 306 simplex AFLP markers, just 16 of the *Ry$_{sto}$* markers were tightly linked together in linkage analysis with LOD grouping thresholds from 3.0 to 10.0. The results of phenotypic evaluation corresponded exactly with STM0003D and six AFLP markers (E+ACC/M+CGC, E+ACC/M+CTC, E+ATC/M+CGCa, P+GAG/M+CAT, P+GAG/M+CGT and P+GAG/M+CGA). If the line P3 was

removed in the linkage analysis, the five other markers (E+AAC/M+CAC, E+ACT/M+CGG, E+ATC/M+CGCb, P+GAC/M+CAT and P+GGC/M+CGA) with a distance of 1.8 cM were also co-segregated. So the line P 3 was detected as a recombinant line.

XII

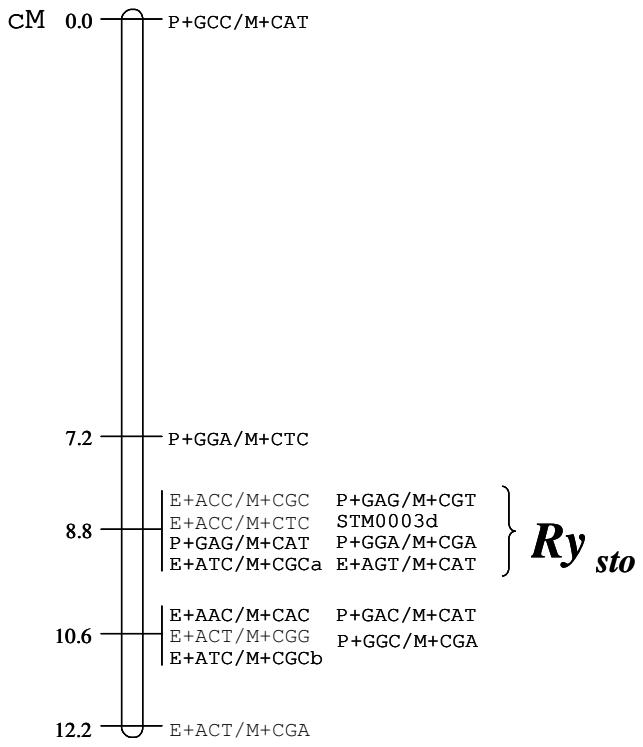


Fig. 9: Linkage group of *Ry*$_{sto}$ markers on chromosome XII

3.1.6 Cloning of *Ry*$_{sto}$ marker

3.1.6.1 *Ry*$_{sto}$ AFLP marker

One *Ry*$_{sto}$ AFLP marker fragment (P+GAC/M+CAT, 199 bp) was eluted from a denatured 4.5 % poly-acrylamide gel and re-amplified using selective primers. Even though only the 199 bp resistance band was eluted, there were more bands beneath the target band after re-amplification. After sequencing the eluted and cloned AFLP fragment, two different sequence data, U5 (199 bp) and U7 (203 bp) were obtained (Fig. 10).

U5

```
           10          20          30          40          50          60
GACTGCGTAC ATGCAGGAGA TAGCAGCCAG AAGGAACAGT AAACACAACT AACAGACGTA
           70          80          90          100         110         120
TATGGTTGAG TTTCATCTAC TGATTGCTAA CTGTTCAAGA TTTTATGGCT GTCGCATGAA
           130         140         150         160         170         180
AGTTGGTCGG TCAGAGCTCA CAAACAGCTT GTGTTGTCGG ATCCTACGTT CAGAGGCTAA
           190         200                     ^^^^^^^^
ATGTTACTCA GGACTCATC                        BamHI
```

U7

```
           10          20          30          40          50          60
GATGAGTCCT GAGTAACATG ACTATTCCCA TGCCCTTTAT CACTTCATCT GTGTAATAAT
           70          80          90          100         110         120
CAGGCTCTGA ATCTTGGAGG GATAGGAGAA CATCAATCAT GGTATTGTTC TTTTGCTCTA
           130         140         150         160         170         180
AAGAAGATCC CTTTCGACGG TTCTTGTGCT CTTCAATCAA TTCTTGCATG AATTTATCCC
               ^^^^^
             TaqI
           190         200         210
TCTTCTCCTG CATGTACGCA GTC
```

Fig. 10: Sequence information of two cloned fragments from one *Ry*$_{sto}$ AFLP marker (P+GAC/M+CAT)

From sequence analysis using the VectorNTI program, the U5 fragment has a *Bam*HI restriction enzyme site while the U7 fragment has a *Taq*I restriction enzyme site. To confirm the correct clone of the *Ry*$_{sto}$ AFLP marker, re-amplified DNA of the eluted fragments marked by fluorescein were digested by *Bam*HI or *Taq*I or both enzymes, and through these reactions U5 was detected as the right one (Fig. 11).

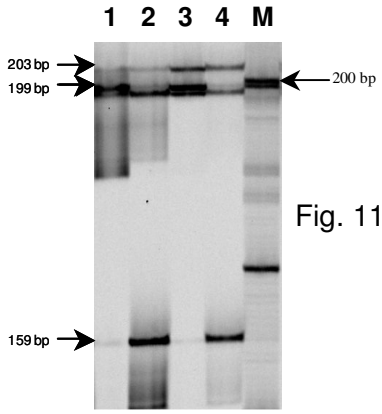


Fig. 11: Restriction analysis of cloned template DNA fragments based on sequence information
Lane 1 *Taq*I digestion, Lane 2 *Bam*HI and *Taq*I digestion, Lane 3 Control, Lane 4 *Bam*HI digestion, M size marker

According to sequence analysis, U5 should have two fragments, 159 bp and 40 bp after being cut with *Bam*HI restriction enzyme. The 159 bp fragment includes a *Pst*I site at the end from the *Pst*I/*Mse*I AFLP reaction, so that it was visible after scanning without post-dying with Vistra Green™ while U7 should have two fragments, 134 bp and 69 bp marked with fluorescence after digestion with the

restriction enzyme *Taq*I. In this gel document, only the fluorescein marked fragments at the 5´end were detected. The National Center for Biotechnology Information (NCBI) had no homologous information of the U5 clone by Basic Local Alignment Search Tool. However, the U7 fragment was analyzed to be homologous to protein cytochrome P450 of *Arabiodopsis thaliana* (NM_126131) scoring 154 (bits 63.9, expectation 4e-010).

3.1.6.2 *Ry$_{sto}$* SSR marker

One fragment of STM0003 was detected as a *Ry$_{sto}$* marker after testing in the dihaploid "Assia" population (Fig. 12) and 110 potato varieties.

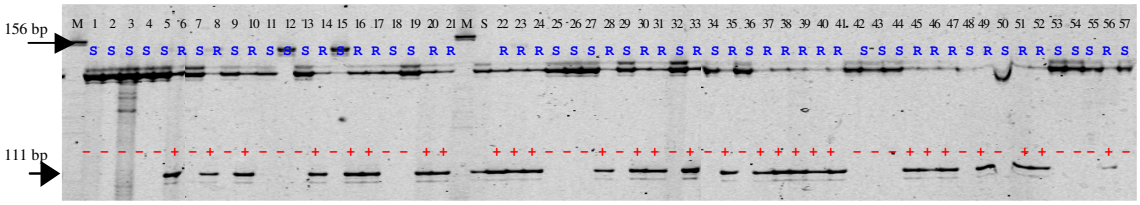


Fig. 12: Segregation of STM0003 in the dihaploid "Assia" population
"R" resistant, "S" susceptible, the fragment (111 bp) indicates the respective polymorphism

The STM0003D marker fragments were eluted from agarose gel and cloned using a TOPO TA Cloning ® kit for sequencing. Eight clones were sequenced and sequence data are presented (Fig. 13).

```
                  10          20          30          40          50          60
clone 1   GGAGAATCAT AACAACCAGC ATATGGTGGA CTATTTGGAG GGAAAGAAAT CCTAGGTGTT
clone 2   GGAGAATCAT AACAACCAGC ATATGGTGGA CTATTTGGAG GGAAAGAAAT CCTAGGTGTT
clone 3   GGAGAATCAT AACAACCAGC ATATGGTGGA CTATTTGGAG GGAAAGAAAT CCTAGGTGTT
clone 4   GGAGAATCAT AACAACCAGC ATATGGTGGA CTATTTGGAG GGAAAGAAAT CCTAGGTGTT
Inverted
clone 5   AATTGTAACT CTGTGTGTGT GTGTGTGT   ATACTATTCT CTATTTTCTC CAACACCTAG
clone 6   AATTGTAACT CTGTGTGTGT GTGTGTGTGT ATACTATTCT CTATTTTCTC CAACACCTAG
clone 7   AATTGTAACT CTGTGTGTGT GTGTGTGTGT ATACTATTCT CTATTTTCTC CAACACCTAG
clone 8   AATTGTAACT CTGTGTGTGT GTGTGTGTGT ATACTATTCT CTATTTTCTC CAACACCTAG
                  70          80          90          100         110         120
clone 1   GGAGAAAATA GAGAATAGTA TACACACACA CACA       GAGTTACAAT T
clone 2   GGAGAAAATA GAGAATAGTA TACACACACA CACACACACA GAGTTACAAT T
clone 3   GGAGAAAATA GAGAATAGTA TACACACACA CACACACACA GAGTTACAAT T
clone 4   GGAGAAAATA GAGAATAGTA TACACACACA CACACACACA GAGTTACAAT T
Inverted
clone 5   GATTTCTTTC CCTCCAAATA GTCCACCATA TGCTGGTTGT TATGATTCTC C
clone 6   GATTTCTTTC CCTCCAAATA GTCCACCATA TGCTGGTTGT TATGATTCTC C
clone 7   GATTTCTTTC CCTCCAAATA GTCCACCATA TGCTGGTTGT TATGATTCTC C
clone 8   GATTTCTTTC CCTCCAAATA GTCCACCATA TGCTGGTTGT TATGATTCTC C
```

Fig. 13: Sequence data of 8 cloned DNA fragments of the STM0003D marker

The sequence information showed that clones 1 to 4 had the same orientation (´5 → 3´) whereas clones 5 to 8 are cloned in an inverted orientation. The insertion size of clone 1 is 105 bp, lacking the $(CA)_3$ repeat sequence in reward primer binding site and clone 5 has 109 bp, lacking one (AC) repeat sequence compared to the others (111 bp). In Simple Sequenced Repeat assays, some minor bands near major bands were frequently produced. A cloned SSR *Ry$_{sto}$* target fragment of STM0003 consisted of polymorphic bands and was analyzed in denaturing poly-acrylamide gel (Fig. 14).

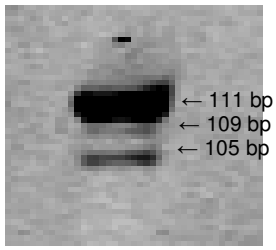


Fig. 14: Enlarged target PCR bands of the target fragment from STM0003

Sequence data and cloned target fragments of STM0003 *Ry$_{sto}$* marker are accorded into each other in three different sizes (111 bp, 109 bp and 105 bp). The produced wavelet bands have almost the same sequences with different numbers of repeats.

3.1.7 Development of a PCR based selection system

Based on U5 sequence data, 3 different PCR primer combinations were designed (Table 16). U5-1 primer was established by the Vector NTI software program. The primers U5-2 and 2-H were designed by segregating data. They include the whole size of the AFLP marker without the core sequence of AFLP selective primers. The 2-H primer was specially designed for a high annealing temperature to make it possible to perform a two-step PCR program consisting of denaturing and annealing & extension. After denaturing at 94°C for 3 min, subsequent cycles at 94°C for 30 s and at 72°C for 40 s were repeated 30 times. The two-step PCR profile took around 80 min.

Table 16: Designed *Ry$_{sto}$* PCR marker

Primer	Size	Annealing T (°C)	Sequence (5´ → 3´)
U5-1	131 bp	59.4	F TGCAGGAGATAGCAGCCAGA
			R TGTGAGCTCTGACCGACCAA
U5-2	175 bp	59.4	F TGCAGGAGATAGCAGCCAGA
		53.0	R TAACATTTAGCCTCTGAAC
2-H	175 bp	72.0	F TGCAGGAGATAGCAGCCAGAAGGAACAGTAAACACAACTAAC
			R TAACATTTAGCCTCTGAACGTAGGATCCGACAACACAAGC

Twenty-four selected lines of the dihaploid “Assia” population were tested with U5-1, U5-2 and 2-H in comparison to one co-segregated AFLP marker (E+AGG/M+CGG, 310 bp) and the STM0003D SSR marker. The results of the PCR test indicate U5-2 and 2-H are suitable for selection of PVY resistant lines (Fig. 15; C).

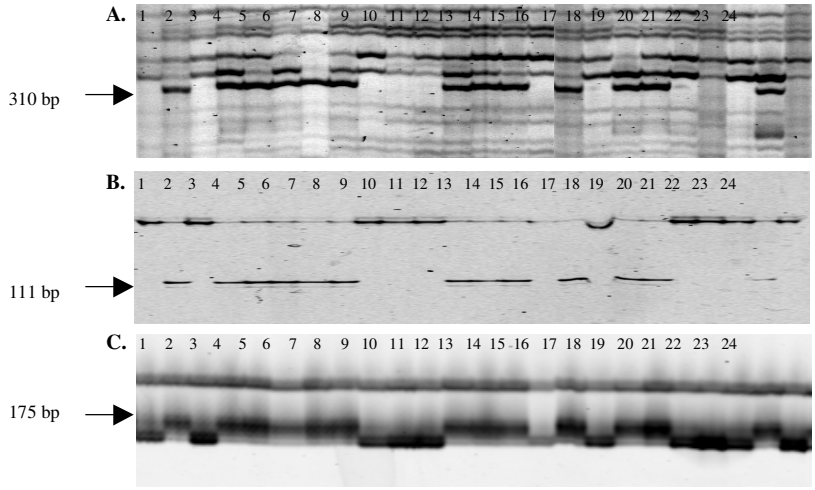


Fig. 15: Comparison of AFLP, SSR and PCR marker segregation in 24 lines of the dihaploid “Assia” population
A. AFLP marker (E+ACT/M+CGG), B. SSR marker, C. developed PCR marker (U5-2)

The PCR marker (primer 2-H) was also applied to 8 potato cultivars using the two-step PCR profile. Based on the above data, 4 resistant and 4 susceptible potato cultivars were tested (Fig. 16). The PCR marker also worked in these tetraploid genotypes but the bands were much weaker than in the dihaploid population.

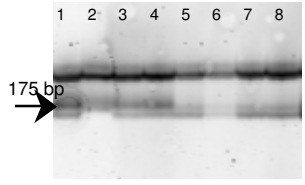


Fig. 16: Test of the *Ry$_{sto}$* PCR marker (2-H) in 8 potato cultivars
Lane 1 to 4 resistant cultivars “Bettina”, “Forelle”, “Ute” and “Kuras” Lane 5 to 8 susceptible cultivars “Laura”, “Panda”, “Sempra” and “Walli”

3.1.8 *Ry$_{sto}$* marker test in cultivated potatoes

For further confirmation, one SSR and 12 AFLP markers were tested in 110 potato cultivars. Nineteen cultivars ("Arosa", "Alwara", "Assia", "Bettina", "Dania", "Forelle", "Franzi" (1977), "Hinga", "Jumbo", "Klepa", "Kuras", "Meduza", "Nimfy", "Oktan", "Petra", "Sibu", "Solara", "Tomba" and "Ute") were detected by observing 8 AFLP markers and one SSR marker as extreme resistant to PVY in alignment with phenotypic reports (Table 17).

The cytoplasma types of cultivated potatoes were added into table 17 based on the data from Dr. Lössl given to the Bayerische Landesanstalt für Landwirtschaft (LfL) in 1997 and in the following internet publication: http://www.flg.tum.de/pbpz/mm/mt/sortzue1.htm. Using an *Eco*RI/*Mse*I assay, two different marker types (A, B) were observed in the cultivated potatoes (Fig. 17). Marker type A detected all cultivars with extreme resistance while marker type B (E+ACC/M+CGC, E+ACT/M+CGG, E+ATC/M+CGC) did not detected "Alwara", "Arosa", "Hinga", "Meduza" and "Nimfy". Four AFLP markers (marker types B and P+GAC/M+CAT) did not recognize all cultivars with extreme resistance. According to the results of 3.1.5, three of these markers are more distant to the *Ry$_{sto}$* locus and could be overcome by recombination.

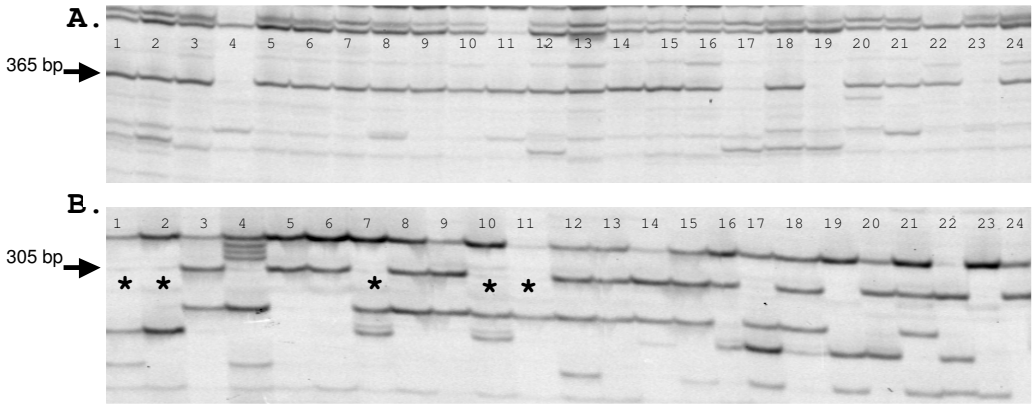


Fig. 17: Two different types of *Ry$_{sto}$* AFLP markers in 24 cultivated potatoes having a high level of PVY resistance
A: *Ry$_{sto}$* AFLP marker (E+ACC/M+CTC), B: *Ry$_{sto}$* AFLP marker (E+ACT/M+CGG), *indicates that these cultivars were not detected by marker type B
lane 1 "Alwara", lane 2 "Arosa", lane 3 "Dania", lane 4 "Fox", lane 5, 6 "Franzi", lane 7 "Hinga", lane 8 "Jumbo", lane 9 "Klepa", lane 10 "Meduza", lane 11 "Nimfy", lane 12 "Oktan", lane 13 "Solara", lane 14 "Bettina", lane 15 "Forelle", lane 16 "Kuras", lane 17 "Laura", lane 18 "Petra", lane 19 "Artis", lane 20 "Sibu", lane 21 "Tomba", lane 22 "Ute", lane 23 "Sempra", and lane 24 "Assia"

Table 17: Analysis of 110 potato varieties with *Ry$_{sto}$* markers

Cultivar	[1]Nation	[2]Usage	[3]Year	[4]Resistance level to PVY	[5]Mitochondira type			[6]*Ry$_{sto}$* AFLP and SSR markers												
								*Pst*I/*Mse*I assay							*Eco*RI/*Mse*I assay						
					[a]1997	[b]Web	[c]LfL	GAC/ CAT	GAG/ CAT	GAG/ CGT	GGA/ CGA	GGA/ CTC	GGC/ CGA	AAC/ CAC	ACC/ CGC	ACC/ CTC	ACT/ CGG	AGT/ CAT	ATC/C GCb	SSR
Acapella	D	Sp	2000	2																
Adora	NL	Sp	1988	6.5 (3.5)																
Agria	D	Sp	1985	2	β	β														
Alwara	D	Sp	1985	1	γ	γ		+	+	+	+	+	+	+	-	+	-	+	-	+
Amigo	D	Wi	1970	7	β	β														
Angela	D	Sp	2000	5																
Arosa	D	Sp	1996	1			γ	+	+	+	+	+	+	+	-	+	-	+	-	+
Artana	D	Wi	1990	6	α	α														
Artis	D	Wi	1997	4																
Assia	D	Wi	1980	1*	γ	γ	γ	+	+	+	+	+	+	+	+	+	+	+	+	+
Astarte	NL	Wi	1975	8.5 (1.5)																
Astoria	D	Sp	1997	2																
Atica	D	Sp	1971	3																
Baronesse	D	Sp	1995	2	α	α														
Bettina	D	Sp	1988	1*	γ	γ	γ	+	+	+	+	+	+	+	+	+	+	+	+	+
Brigit	D	Sp	1979	2	α	α														
Bolero	D	Sp	1998	1																
Bonanza	D	Wi	1993	1	α	α	α													
Calla	D	Wi	1990	2	α	α														
Camilla	D	Sp	1998	1																
Carmona	D	Sp	2000	2																
Ceza	Po	Wi	1986																	
Christa	D	Sp	1975	6	β	β														
Colette	D	Sp	1995	1	α	α														
Combi	D	Wi	1988	1	α	α	α													
Dania	D	Sp	2001	1	γ	γ	γ	+	+	+	+	+	+	+	+	+	+	+	+	+
Debora	D	Sp	2000	4																
Desiree	D	Sp	1962	2	β	β	β													
Ditta	D	Sp	1991	2	β	β														
Ekra	Po	Wi	1991																	
Elkana	NL	Wi	1976	7.5 (2.5)																
Exempla	D	Sp	1997	3																
Exquisa	D	Sp	1992	2	β	β														
Fausta	D	Wi	1976	3	α	α														
Festien	D	Wi	2000	1																
Flora	D	Sp	1998	2																
Forelle	D	Sp	1979	1*	γ	γ	γ	+	+	+	+	+	+	+	+	+	+	+	+	+
Fox	D	Wi	1981	1	γ	γ														
Franca	D	Wi	1987	3	α	α														
Franzi	D	Sp	1977	1*	γ	γ	γ	+	+	+	+	+	+	+	+	+	+	+	+	+
Franzi	D	Sp	2001	2			α													
Frieda	D	Sp	1999	3																
Gloria	D	Sp	1972	6	β	β														
Granola	D	Sp	1975	3	β	β														
Gunda	D	Sp	1999	5																
Hansa	D	Sp	1956	8	β	β														
Hinga	Po	Sp	1996				γ	+	+	+	+	+	+	+	-	+	-	+	-	+
Indira	D	Wi	1981	2	β	β														
Impala	D	Sp	1941	4	β	β														
Irmgard	D	Sp	1960	4																
Jetta	D	Sp	1967	4	β	β														
Juliane	D	Sp	1999	2																
Jumbo	D	Wi	2002	1*			γ	+	+	+	+	+	+	+	+	+	+	+	+	+
Kardent	NL	Sp	1995	7.5 (2.5)																
Kartel	NL	Sp	1993	4.5 (6.5)																

[1]Nation : D. Germany, NL. The Netherlands, Po. Poland, [2]Usage: Sp. = ware potatoes, Wi. = economic usage, [3] Year of registered, [4]Resistance level to PVY: 1 (high resistant) to 9 (high susceptible) in Germany, 9 (high resistant) to 1 (high susceptible) in The Netherlands, 1*: Extreme resistance to potato virus Y registration in Bundessortenamt, [5] The mitochondria type based on the data, [a]1997 from Dr. Lössl given to LfL on 1997, [b]Web from http://www.flg.tum.de/pbpz/mm/mt/sortzue1.htm and [c]LfL from own results, [6]*Ry$_{sto}$* AFLP and SSR markers: + = band present, - = band absent, +/- = unclear to score the band

Table 17 continued

Cultivar	[1]Nation	[2]Usage	[3]Year	[4]Resistant level to PVY	[5]Mitochondira type [a]1997	[b]Web	[c]LfL	[6]*Ry*$_{sto}$ AFLP and SSR markers *Pst*I/*Mse*I assay GAC/CAT	GAG/CAT	GAG/CGT	GGA/CGA	GGA/CTC	GGC/CGA	*Eco*RI/*Mse*I assay AAC/CAC	ACC/CGC	ACC/CTC	ACT/CGG	AGT/CAT	ATC/CGCb	SSR
Klepa	Po	Wi	1997				γ	+	+	+	+	+	+	+	+	+	+	+	+	+
Kuras	NL	Wi	1996	9.5 (0.5)	γ	γ	γ	+	+	+	+	+	+	+	+	+	+	+	+	+
Laura	D	Sp	1998	1																
Likaria	D	Sp	1986	5	β	β														
Liseta	D	Sp	1987	9.5 (0.5)																
Marabel	D	Sp	1993	1	α	α														
Marella	D	Sp	2001	1																
Maxilla	D	Wi	1981	2	α	α														
Meduza	Po	Wi	1997				γ	+	+	+	+	+	+	+	-	+	-	+	-	+
Miriam	D	Sp	1988	3	β	β														
Mondial	NL	Sp	1987	7.5 (2.5)																
Moni	D	Wi	1972	2	α	α														
Monza	D	Sp	1980	1																
Natalie	D	Wi	1995	2	α	α														
Nimfy	Po	Wi	1998	extreme R.			γ	+/-	+	+	-	+	+	+	-	+	-	+	-	+
Nora	D	Sp	2000	1																
Oktan	D	Wi	2000	1			γ	+	+	+	+	+	+	+	+	+	+	+	+	+
Pallina	D	Wi	1995	4	α	α														
Panda	D	Wi	1986	3	α	α														
Pasja	Po	Wi	2000				γ													
Patrona	D	Wi	1994	5	α															
Petra	D	Wi	1991	1	γ	γ	γ	+	+	+	+	+	+	+	+	+	+	+	+	+
Ponto	D	Wi	1984	3	α	α														
Power	D	Wi	1999	3																
Presto	D	Sp	2001	1																
Producent	D	Wi	1986	6	β	β														
Quarta	D	Sp	1979	4																
Rebecca	D	Wi	1984	2	β	β														
Renate	D	Sp	1993	3	α	α														
Rikea	D	Sp	1984	2	α	β														
Rita	D	Sp	1988	2																
Rincona	D	Sp	1999	2																
Ruta	Po	Sp	1985																	
Salome	D	Sp	2001	1																
Satina	D	Sp	1993	1	β	β														
Saturna	D	Sp	1970	6	β	β														
Scala	D	Sp	1982	2																
Selma	D	Sp	1972	3	β	β														
Sempra	D	Wi	1997	2																
Seresta	NL	Wi	1993	7.0 (2.0)																
Sibu	D	Wi	1993	1	γ	γ	γ	+	+	+	+	+	+	+	+	+	+	+	+	+
Sieglinde	D	Sp	1953	3		β														
Sjamero	D	Wi	1997	1																
Solara	D	Sp	1989	1	β	β	γ	+	+	+	+	+	+	+	+	+	+	+	+	+
Sommergold	D	Wi	1987	1	β	β														
Spunta	NL	Sp	1969	7.5 (2.5)																
Terra	D	Wi	2001	1																
Tessi	D	Sp	1998	2																
Tomba	D	Wi	1995	1	γ	β	γ	+	+	+	+	+	+	+	+	+	+	+	+	+
Ukama	D	Sp	1977	6	β	β														
Ulla	D	Sp	1970	8	β	β														
Ulme	D	Wi	1991	1	α	α														
Ute	D	Wi	1982	1*	γ	γ	γ	+	+	+	+	+	+	+	+	+	+	+	+	+
Velox	D	Sp	1994	4	β	β														
Walli	D	Wi	1993	3	β	β														

Two markers (E+ATC/M+CGCa, E+ATC/M+CGCb) were detected in the selective primer amplification E+ATC/M+CGC. Only the E+ATC/M+CGCb marker was co-segregating with PVY resistance in the cultivated potatoes. The polish cultivar “Nimfy” showed a few differences in marker reaction because the target band was unclear with the marker P+GAC/M+CAT and it reacted negatively with the marker P+GGA/M+CGA.

3.1.9 Pedigree analysis of PVY resistant potato cultivars selected with *Ry_{sto}* marker

In total, 19 cultivated potatoes were detected from 110 cultivars by 13 *Ry_{sto}* AFLP and SSR markers and the maternal pedigrees of 18 cultivars were analyzed by using databases from the department of potato breeding at the Bayerische Landesanstalt für Landwirtschaft in Germany, from the private breeding station Böhm-Nordkartoffel Agrarproduktion OHG in Germany, from the internet potato database http://www.dpw.wau.nl/pv/ of The Netherlands and from the Plant breeding and acclimatization institute (IHAR) in Poland in order to track the resistance gene source (Fig. 18). No pedigree data were available for the cultivar “Dania” (renamed “Rania”). Three groups with different genetic background could be distinguished. The first group was derived from the crossing of *S. stoloniferum* with “Erika”. The 7 cultivars “Franzi” (1977), “Alwara” (1985), “Bettina” (1988), “Solara” (1989), “Arosa” (1996), “Meduza” (1989) and “Klepa” (1997) belong to this group. Cultivars “Hinga” (1996) and “Nimfy” belong to the second group, which is derived from the cross between stoXIIB and “Polonia”. The third group has its origin in the crossing parents ((stohybrid) x Frühmolle) x Falke) and Frühmolle. Cultivars “Forelle” (1979), “Assia” (1980), “Ute” (1982), “Petra” (1991), “Sibu” (1993), “Tomba” (1995), “Kuras” (1996), “Oktan” (2000), “Jumbo” (2002) belong to this third group.

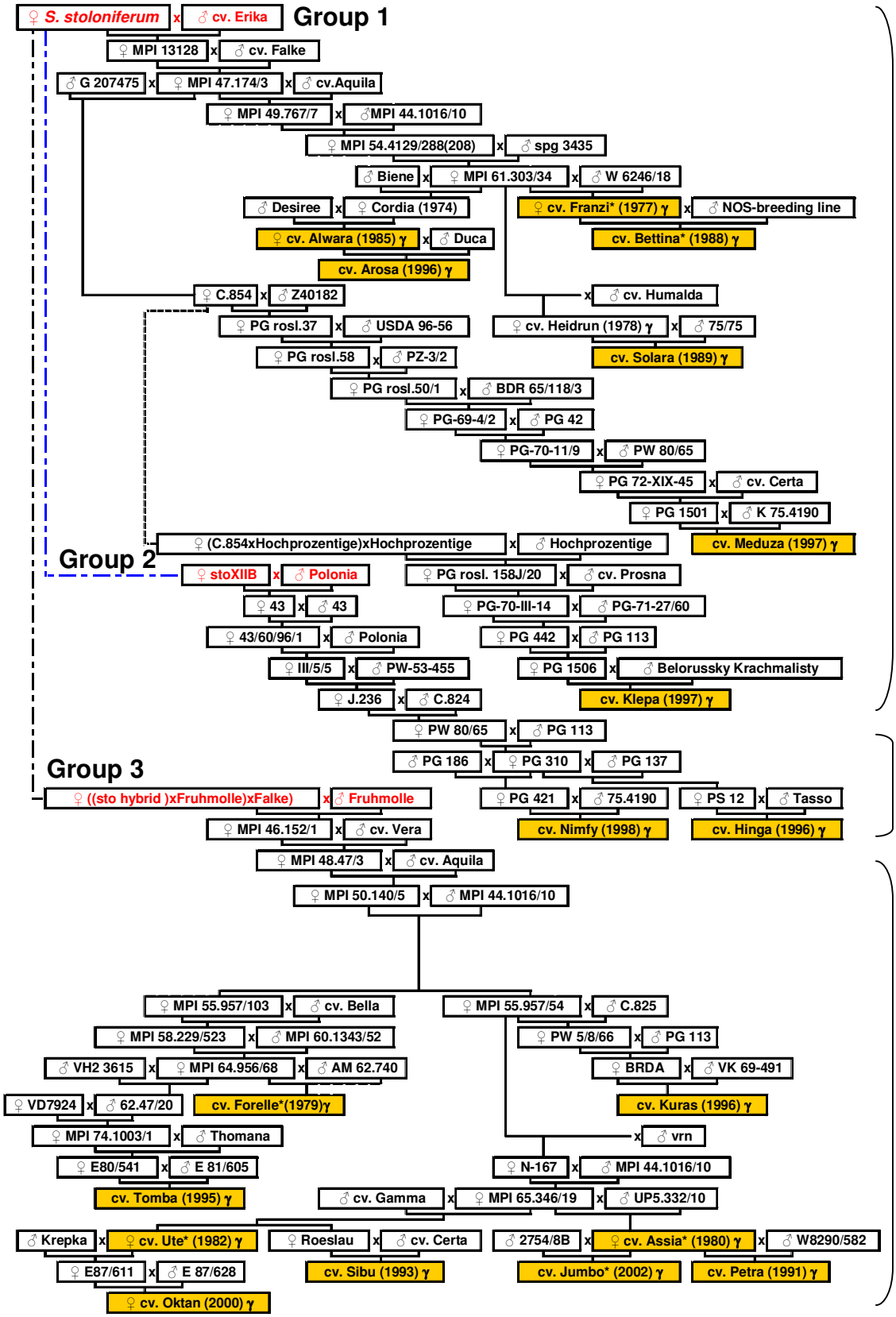


Fig. 18: The maternal pedigree of 18 extreme resistant cultivars
Cultivars in yellow boxes were detected by AFLP and SSR marker assisted selection

3.2 Chip color quality after long term storage at 4°C

3.2.1 Evaluation of chip color quality and related traits

3.2.1.1 Chip color segregation

Chip color tests were performed from 1998 to 2001 with the primary dihaploid population of "Artis" after long term (5 to 7 months) storage at 4°C. In the first year, 1998, tubers of 37 randomly selected individuals were fried in order to estimate the chip color segregation within the population. The chip color values had a mean of 4.54 on a scale of 1 to 9. The W value from the normality test was 0.93 and the p value (Pr<W) was 0.0004 based on the Shapiro-Wilk test. The skewness of chip color in 1998 was 0.29 and kurtosis –0.96 with a significance level of $p<0.0001$. Results from the first year demonstrated the utility of this dihaploid population to analyze QTL for chip quality after long term storage at 4°C. During all 4 years, chip colors from dark brown to light yellow (Fig. 19) were obtained within the population.

Fig. 19: Various chip colors in the dihaploid "Artis" population after long term storage at 4°C
Note 1 (dark brown color) to note 9 (light yellow color)

In 2001, the dihaploid "Artis" population was planted in two different field trials, "Schlüter" and "Pulling" with three repeats per field trial. Most of the individuals showed stable chip color. This trait is therefore more affected by genetic than environmental factors (Fig. 20 & 21).

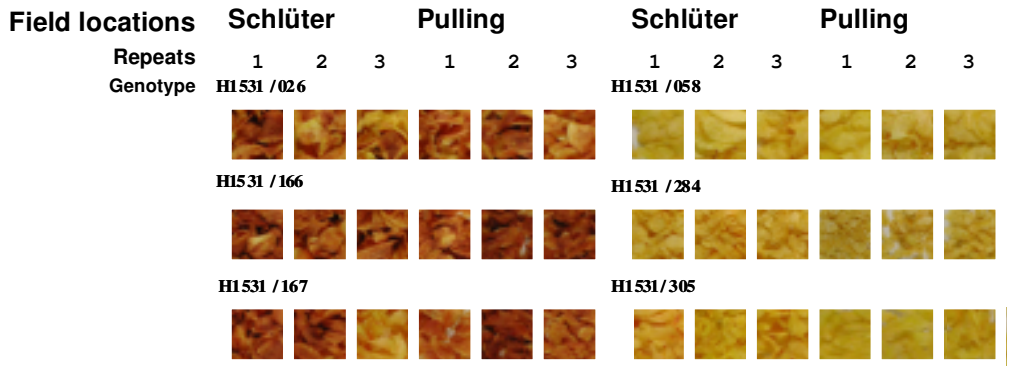


Fig. 20: Reproductive chip colors of 6 individuals in 3 repeats of 2 different field locations in 2001

Fig. 21: Chip color segregation of the dihaploid “Artis” population after long term (6 to 7 months) storage at 4°C in 2001

* Chip color standard cards from Wageningen. A,B indicates two different field trials (Schlüter, Pulling) and 1,2,3 indicates repeat number

The statistical parameters of the chip color datasets from 1999 to 2001 are shown in Table 18.

Table 18: Statistical parameters of chip color datasets in the dihaploid “Artis” population

Parameter	**1999**	**2000**	**2001**	
			Schlüter	Pulling
Mean*	4.55	4.73	4.44	4.88
Median*	4	5	4	5
Standard deviation*	2.43	2.28	2.04	1.96
Skewness	0.26	−0.10	0.16	−0.25
Kurtosis	−1.04	−1.24	−0.76	−0.56
Shapiro-Wilk: W value	0.93	0.93	0.95	0.95
p Value (Pr<W)	0.0004	0.0004	0.0075	0.0351

* Chip color note from 1 to 9

The frequency distribution of the mean chip color in the dihaploid “Artis” population showed significantly a normal distribution (Fig. 22) according to the criteria of the Shapiro-Wilk test. The W value of chip color in the dihaploid “Artis” population was 0.96, the p-value 0.02, and the trait showed continuous variation.

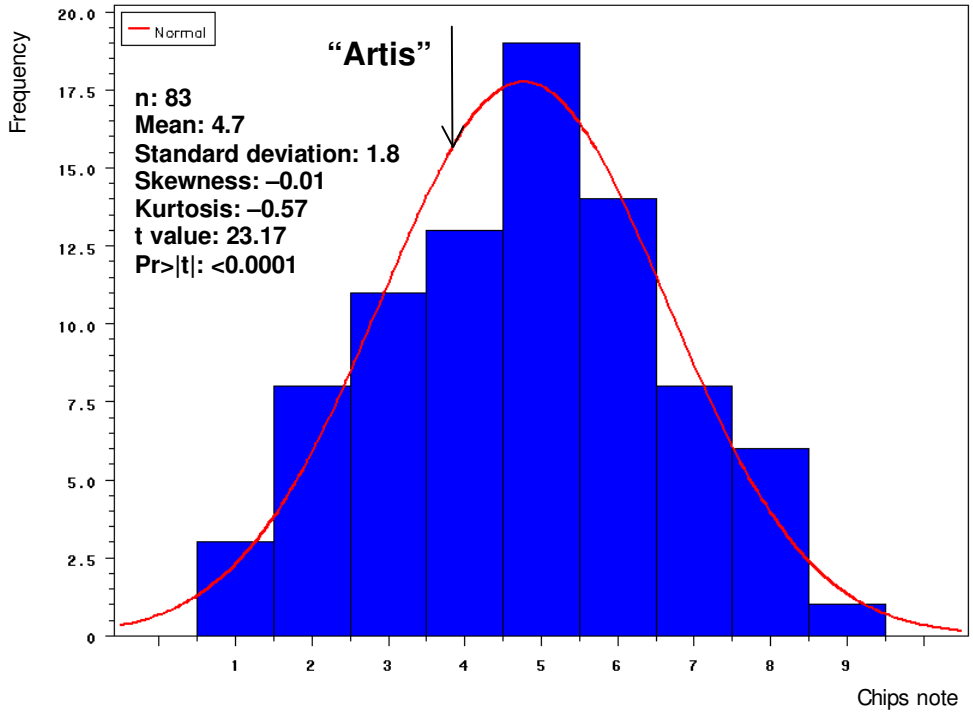


Fig. 22: Frequency distribution of mean chip color in the dihaploid “Artis” population
Note 1 (Dark brown color) to note 9 (light color)

3.2.1.2 Reducing sugar content and chip color

Statistical parameters for the reducing sugar content after long term storage at 4°C are summarized in Table 19. The obtained values for glucose and fructose content ranged wide from 0.098 to 8.42 mg/100 mg dry matter and from 0.04 to 11.08 mg/100 mg dry matter.

Table 19: Statistical parameters of reducing sugar content in the dihaploid "Artis" population

Parameter	**Glucose** (mg/100mg dry matter)	**Fructose** (mg/100mg dry matter)
Mean	1.47	2.26
Median	0.95	1.70
Standard deviation	1.71	2.18

Glucose and fructose content of tubers were correlated highly with each other (r= 0.94) based on Pearson correlation coefficient analysis (Fig. 23).

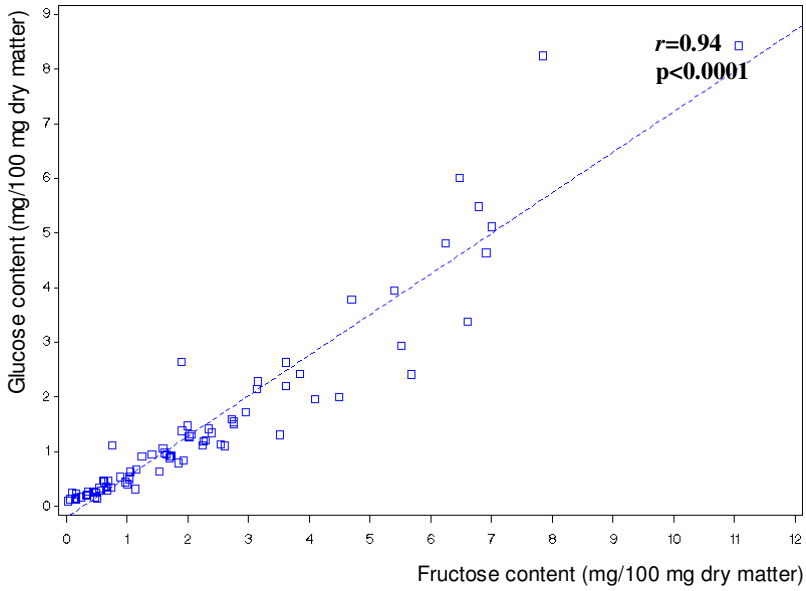


Fig. 23: Correlation between glucose and fructose content in the dihaplod "Artis" population after long term storage at 4°C

After long term storage at 4°C, chip color is correlated negatively to glucose content (r=-0.67) and fructose content (r=-0.73) with a significance level of p<0.0001 according to Pearson correlation analysis (Fig. 24). The higher the reducing sugar content in the tuber, the darker is the chip color after long term storage at 4°C.

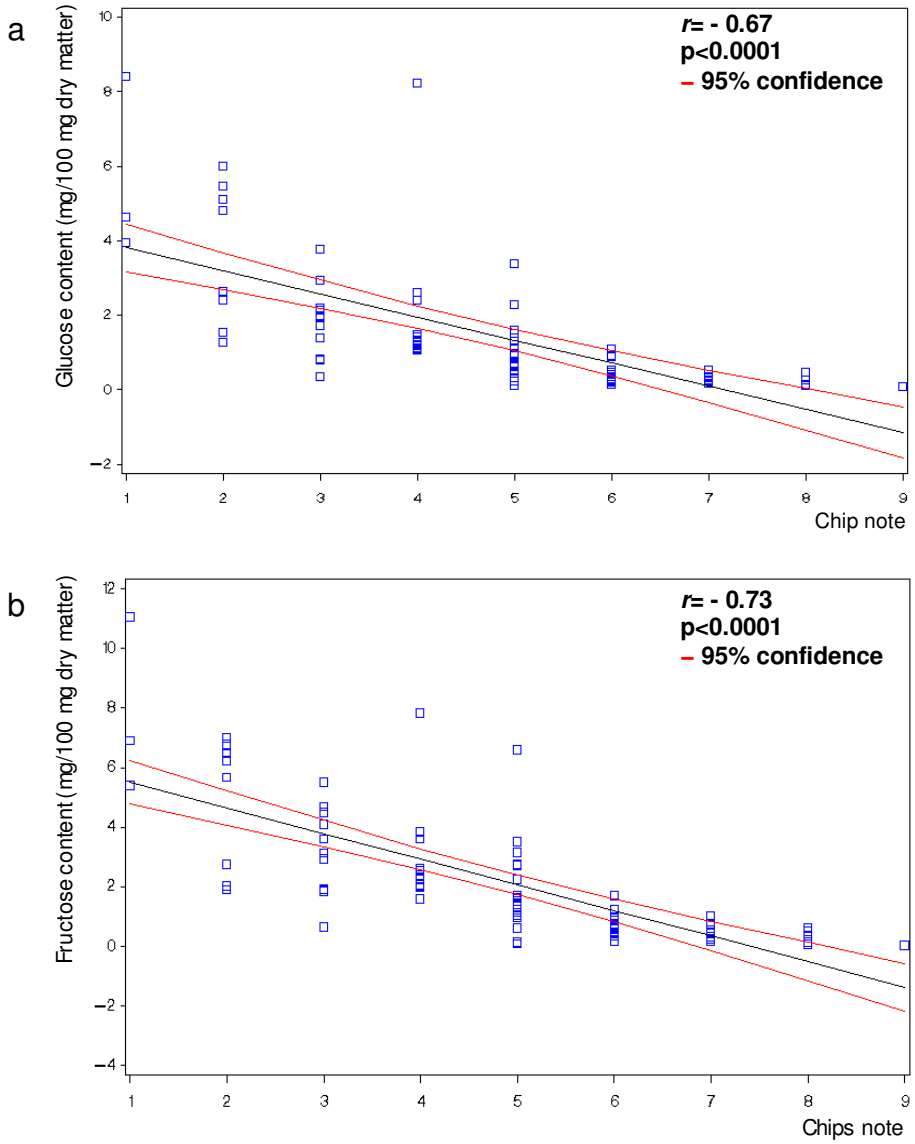


Fig. 24: Linear regression analysis between reducing sugar (a. glucose, b. fructose) content and chip color in the dihaploid "Artis" population after long term storage at 4°C

In linear regression analysis, the phenotypic variance R^2 between glucose content and chip color is 0.46 with a significance of p<0.0001, whereas the phenotypic variance R^2 between fructose content and chip color is 0.54. In conclusion, fructose content has slightly more influence to chip color than glucose content.

3.2.1.3 Starch content and chip color

Before long term storage in 2000, the starch content of 83 dihaploid individuals was measured. The starch content of the cultivar "Artis" was approximately

18.6% after harvest, whereas the starch content of the dihaploid “Artis” population had a mean of 14.7%, i.e. 3.9 % lower than “Artis”. According to the criteria of the Shapiro-Wilk test, starch content in the dihaploid “Artis” population had a W value of 0.97 with a significance level of $p<0.05$. The frequency distribution of starch content in the dihaploid “Artis” population showed a significantly normal distribution (Fig. 25) and continuous variation.

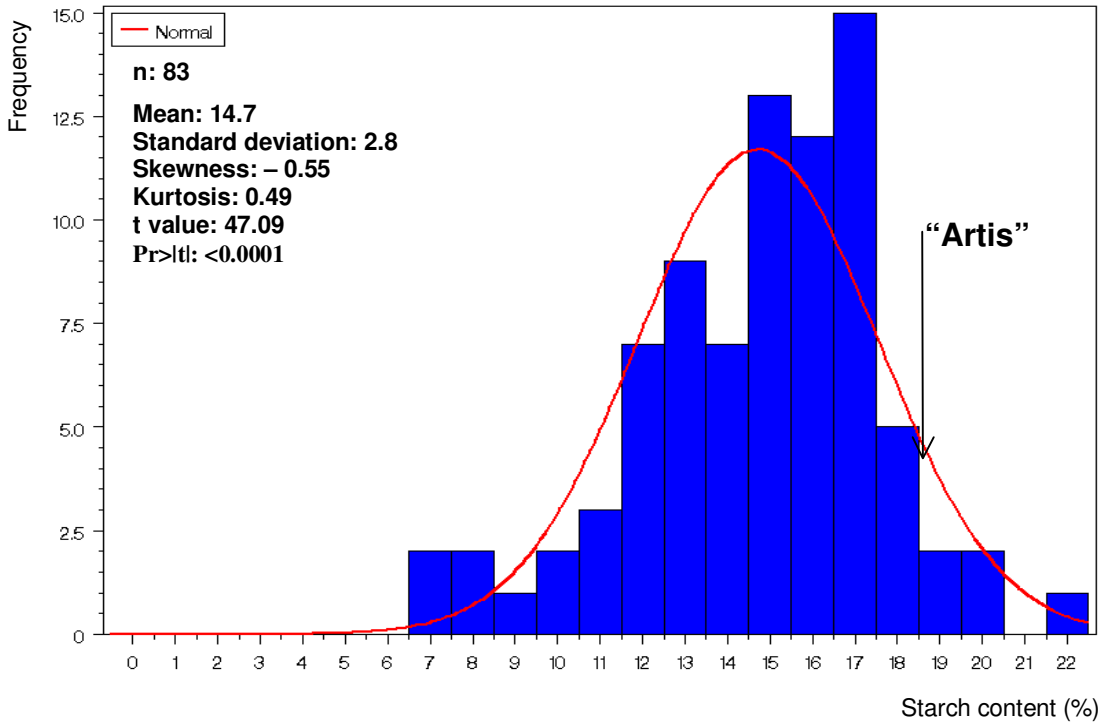


Fig. 25: Distribution of starch content in the dihaploid “Artis” population before storage

After long term storage at 4°C, the starch content of the dihaploid population was measured again. The starch content after storage had a mean of 17.8 % approximately 3.1 % higher than before storage. There was no correlation between different starch content values and chip color or reducing sugar content.

3.2.2 Molecular marker analysis of the dihaploid population

3.2.2.1 AFLP analysis

Clearly visible polymorphisms were obtained with the applied 108 primer combinations. In total, simplex constitution was found in 52.9 % of the 3507

segregating markers in both, i.e. *Eco*RI/*Mse*I, *Pst*I/*Mse*I, assays based on an X^2 test (Table 20).

Table 20: Number of polymorphisms in AFLP analysis per restriction enzyme and primer combination

	Nr. of polymorphism								
	***Eco*RI**			***Pst*I**			**Total**		
Segregation	Total		per [1]p.c.	Total		per [1]p.c.	Total		per [1]p.c.
Simplex	1554	(55.1 %)	17.5	300	(43.6 %)	15.8	1854	(52.9 %)	17.2
skewed simplex	366	(13.0 %)	4.1	122	(17.7 %)	6.4	488	(13.9 %)	4.5
Duplex	601	(21.3 %)	6.8	186	(27.0 %)	9.8	787	(22.4 %)	7.3
skewed duplex	298	(10.6 %)	3.4	80	(11.6 %)	4.2	378	(10.8 %)	3.5
Total	2819		31.7	688		36.2	3507		32.5

[1]p.c stands for primer combination

Duplex was ranked second with around half (22.4 %) of simplex. The remaining polymorphisms showed skewed segregation. Simplex and skewed simplex markers were used for linkage analysis.

3.2.2.2 SSR analysis

In total, 42 polymorphisms were obtained with the 27 published SSR primers. Maximal 4 alleles per primer pair could be observed in the dihaploid population. However, mostly 2 alleles were represented. Four primer pairs (STM0001, STM0010, STM0013 & STM0024) delivered no polymorphism. From the 42 polymorphisms, 31 showed simplex, 2 skewed simplex and 9 duplex constitution.

3.2.2.3 RFLP analysis

Based on the probe screening results, RFLP analysis was performed on the whole population restricted with three different enzymes: *Dra*I, *Eco*RI or *Eco*RV and radioactive labeled GP probes and CP probes. Eighteen simplex and 7 duplex constitutions were demonstrated and over one half of the total polymorphisms showed a skewed segregation in a X^2 test.

3.2.3 Linkage grouping

The dihaploid population was composed of 6 different gamete types from the tetraploid “Artis” that segregated in simplex and duplex, when a single locus is considered with two alleles *A/a* and *A* is dominant to *a* in the dominant marker system of AFLP analysis. The simplex segregation type was alternatively coded

as *Aa* or *aa* and a F_1 backcross population mode was applied to the dihaploid population. In total, 1854 simplex AFLP markers and 488 skewed simplex markers were used to construct a linkage map with 33 SSR and 18 RFLP markers. The markers from the *Pst*I/*Mse*I AFLP assay showed an increase in distorted segregations and were more ungrouped in linkage analysis than markers from the *Eco*RI/*Mse*I AFLP assay (Table 21).

Table 21: Number of unlinked polymorphic markers in AFLP analysis per restriction enzyme and primer combinations

	AFLP						SSR		RFLP	
	*Eco*RI		*Pst*I		Total					
Segregation	Total	unlinked	Total	unlinked	Total	unlinked	Total	Unlinked	Total	unlinked
Simplex	1554	47	300	60	1854	107	31	6	6	0
skewed simplex	366	97	122	53	488	150	2	0	12	0
Total	1920	144	422	113	2342	257	33	6	18	0

From the first primitive linkage map, some co-segregating markers or markers having missing values were removed. In total, 57 linkage groups could be assigned (Fig. 26). Linkage group VIII could not be detected due to lack of simplex anchor markers. Twenty-five linkage groups covering 1379 cM could be assigned to a distinct chromosome number based published potato reference maps (Gebhardt *et al*., 1991; Milbourne *et al*., 1998). 32 further linkage groups covering 1679 cM could not be associated with a certain chromosome number. However, a few AFLP markers (red colored with ´#´) could be aligned with the Ultra High Density map from Wageningen so that the linked chromosome could be identified (Table 22).

Table 22: Marker alignment between dihaploid Artis linkage groups and UHD catalogue

Artis dihaploid population linkage analysis[1]			Ultra High Density catalogue[2]		
Linkage group	Map position (cM)	Marker	Primer combination	Size (bp)	Linked chromosome
LG 2	37	E+ATG/M+CTC_2	E+ATG/M+CTC	566	XII
LG 4	22	E+AAC/M+CCA_20	E+AAC/M+CCA	216 .8	X
LG 5	80	E+AAC/M+CAG_30	E+AAC/M+CAG	205	I
LG 8	27	E+ACA/M+CAC_32	E+ACA/M+CAC	148 .8	IV
LG 17	44	E+AAC/M+CAG_34	E+AAC/M+CAG	188	I
LG 20	61	E+ATG/M+CTC_25	E+ATG/M+CTC	138	XI

[1] result was performed in this study, [2] data from internet http://www.dpw.wau.nl/uhd/

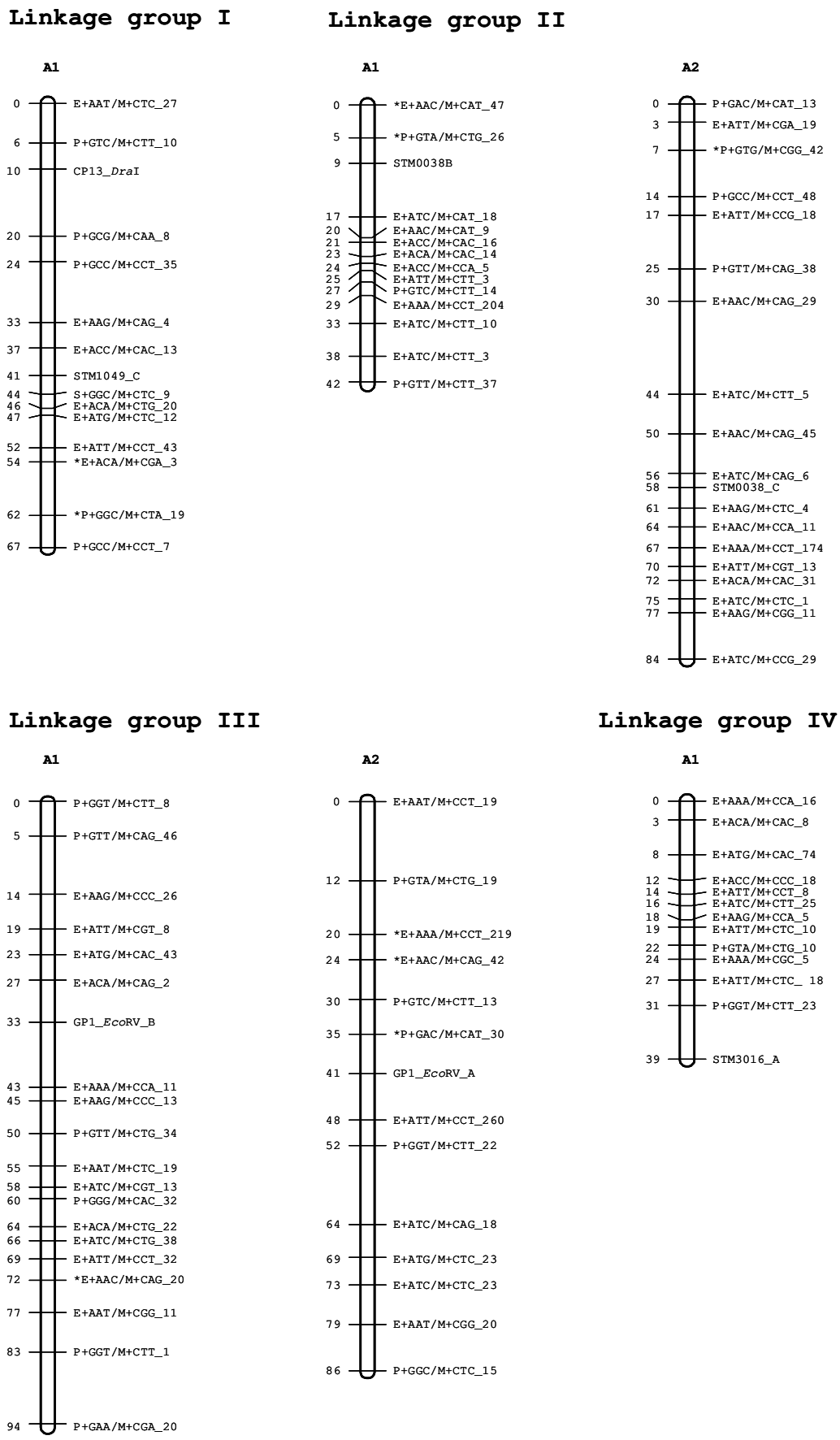
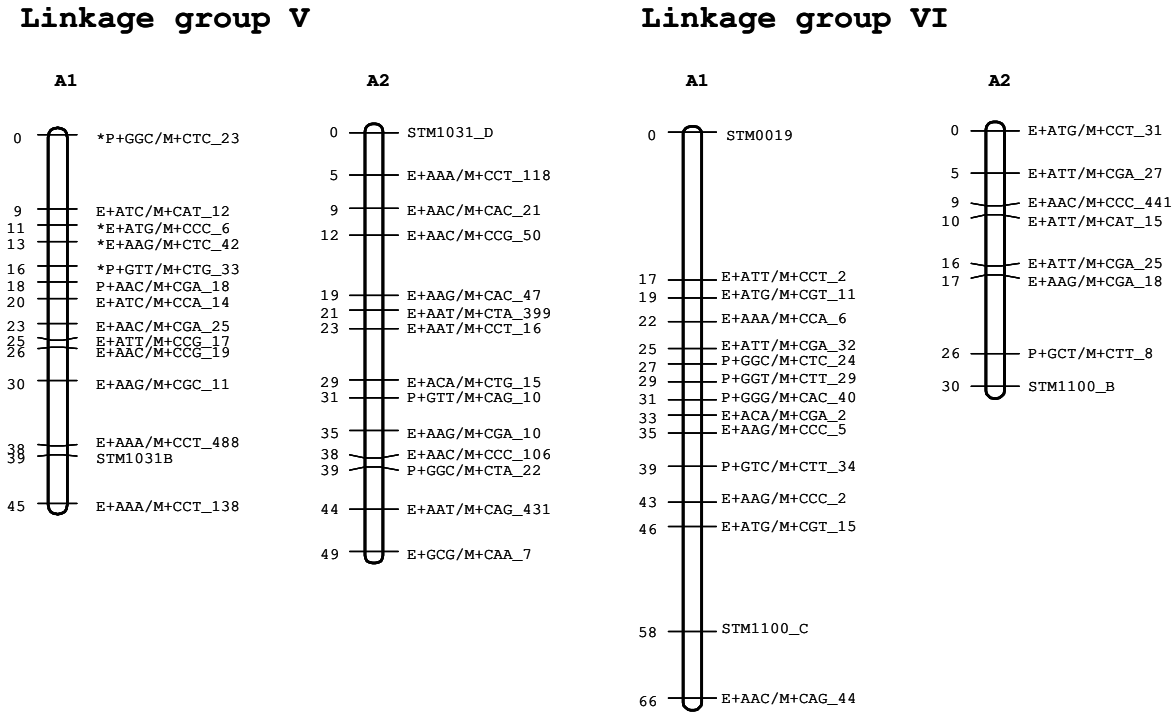


Fig. 26: Genetic linkage map of the primary dihaploid population from “Artis”
* indicates distorted simplex marker in the linkage map and # (red color) marked markers were aligned with the UHD (Ultra High Density) map.



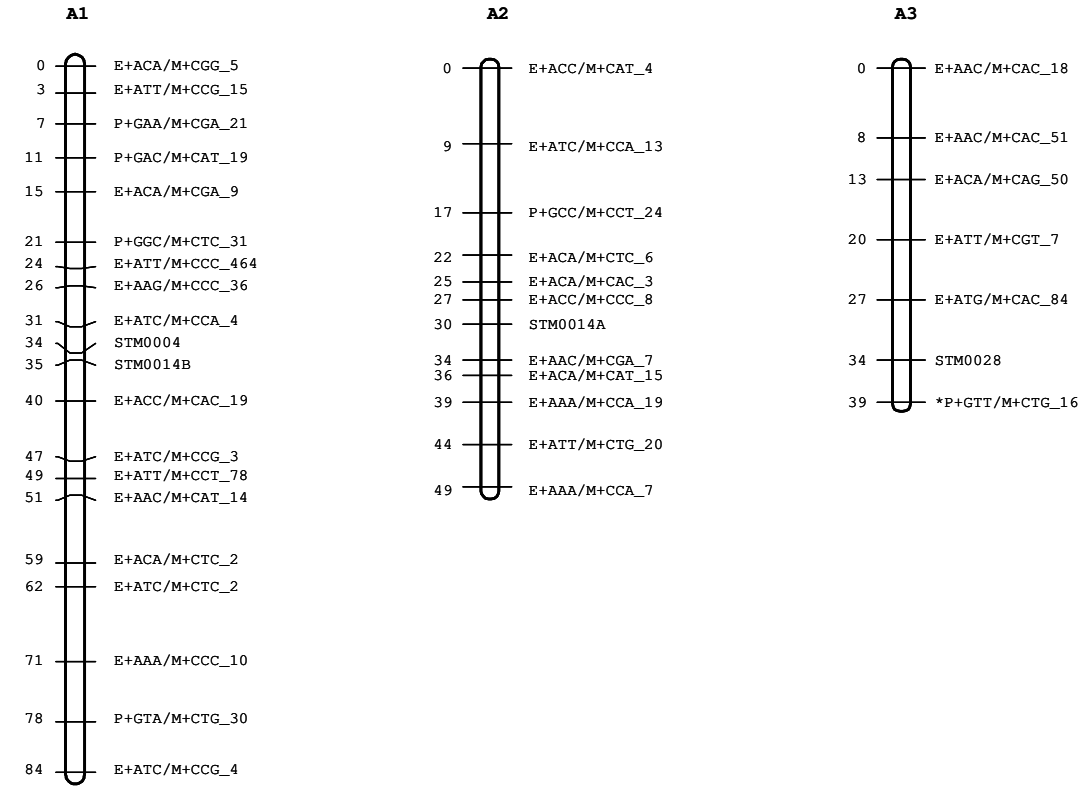
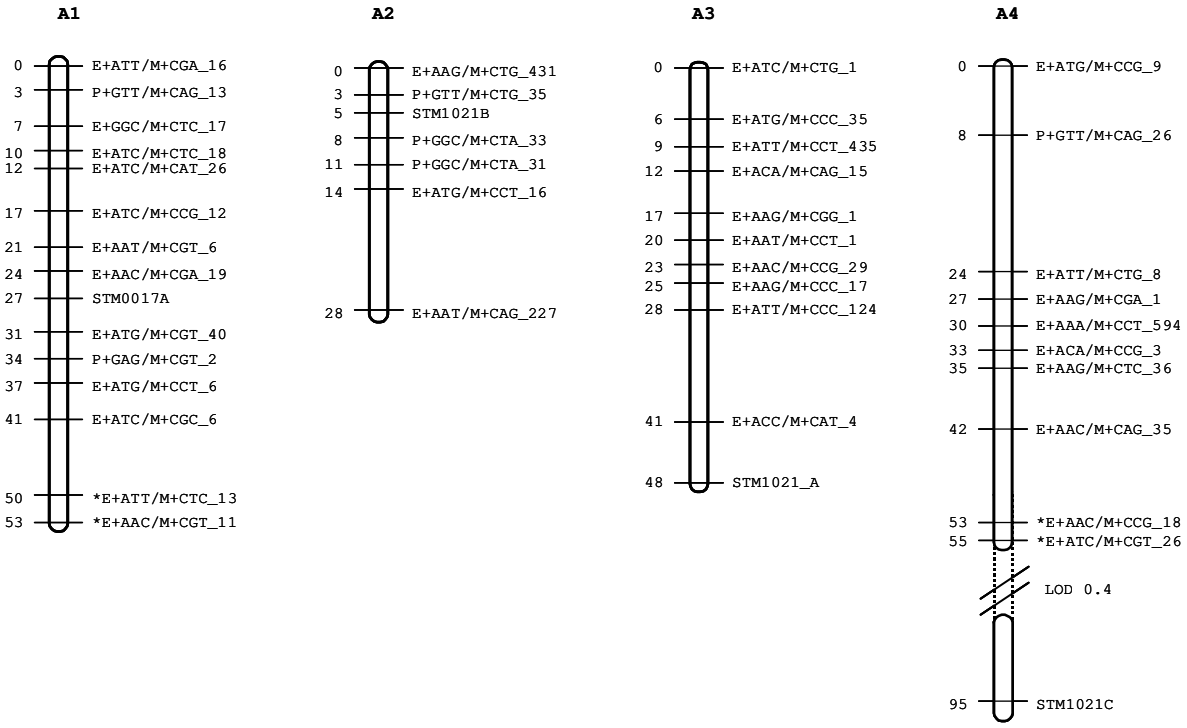


Fig. 26 continued

Linkage group IX



Linkage group X

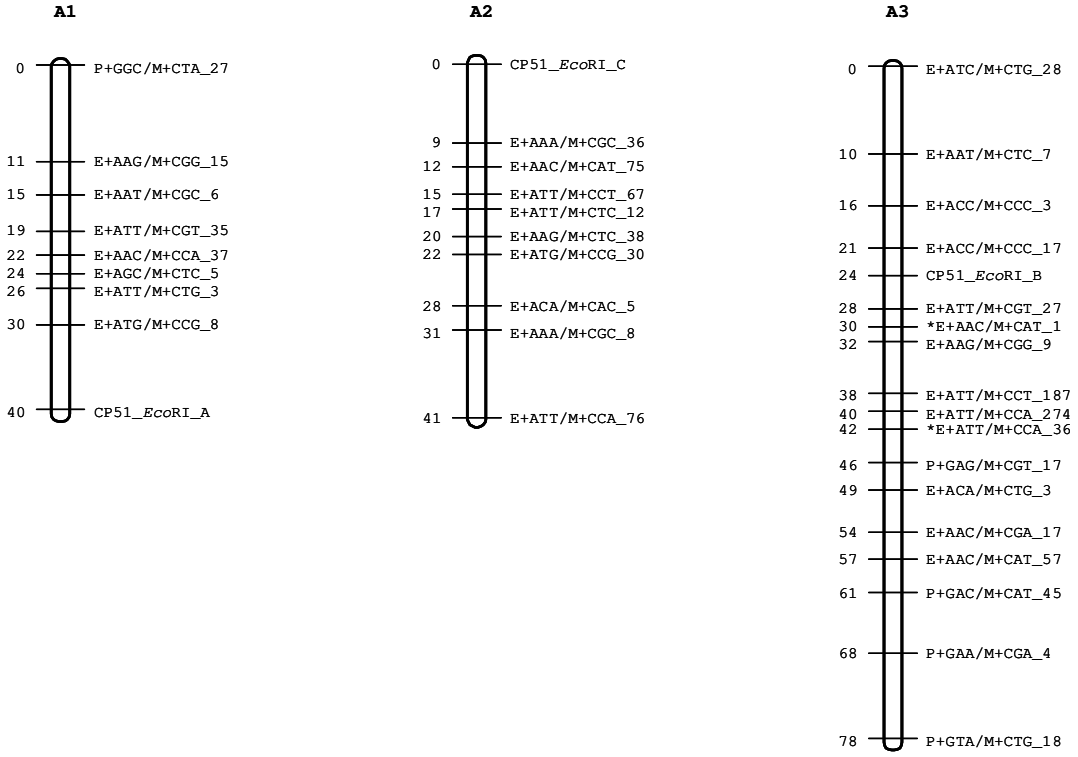
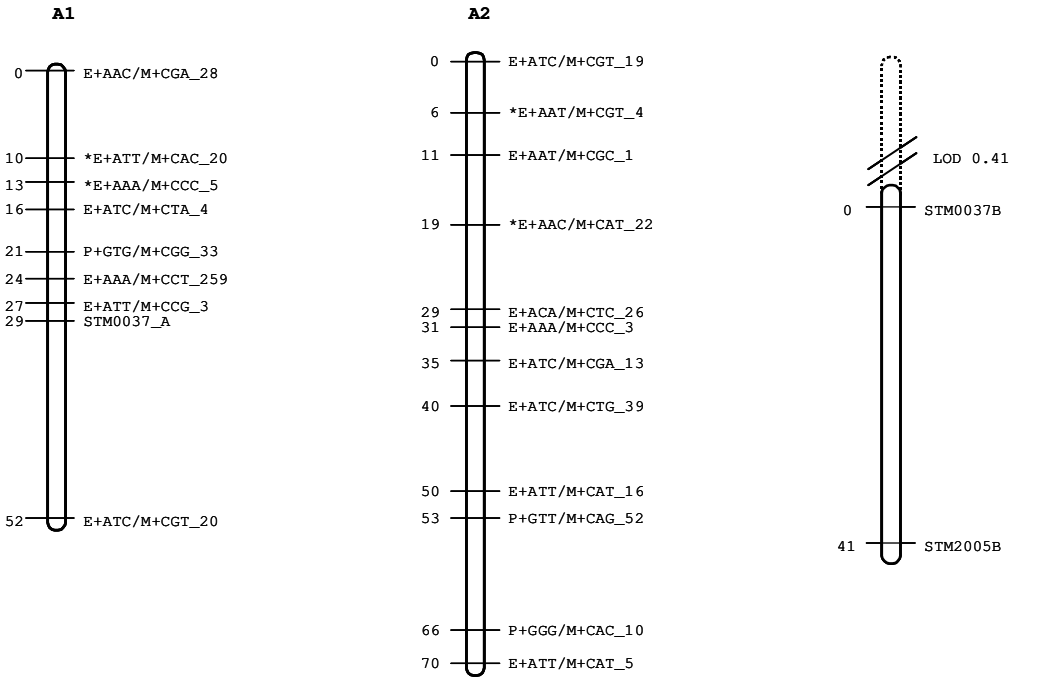


Fig. 26 continued

Linkage group XI



Linkage group XII

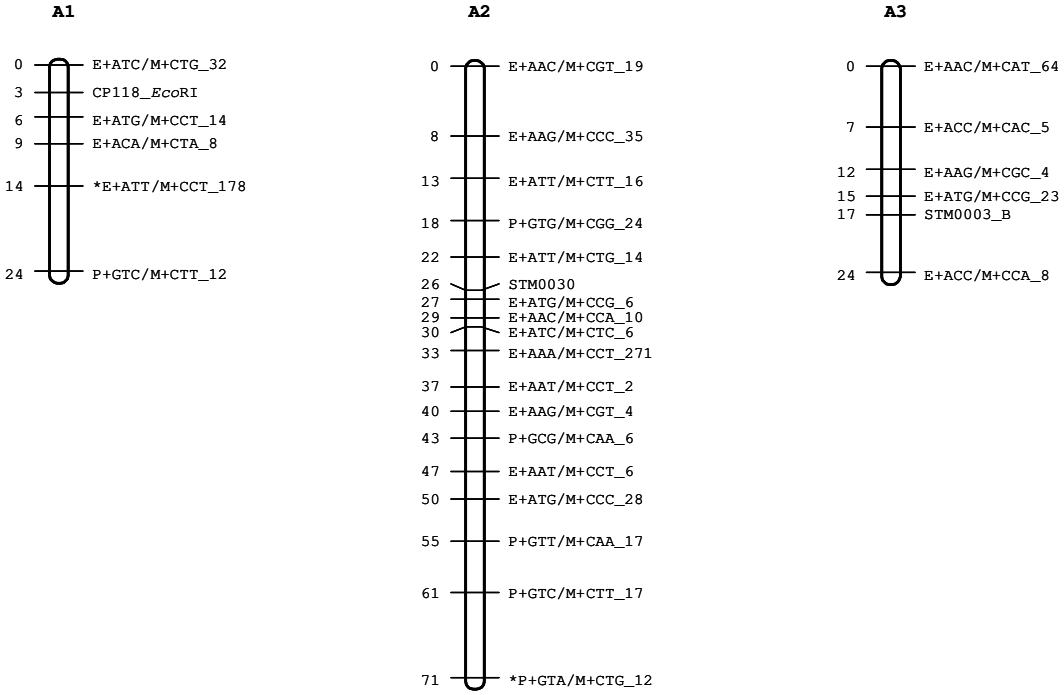


Fig. 26 continued

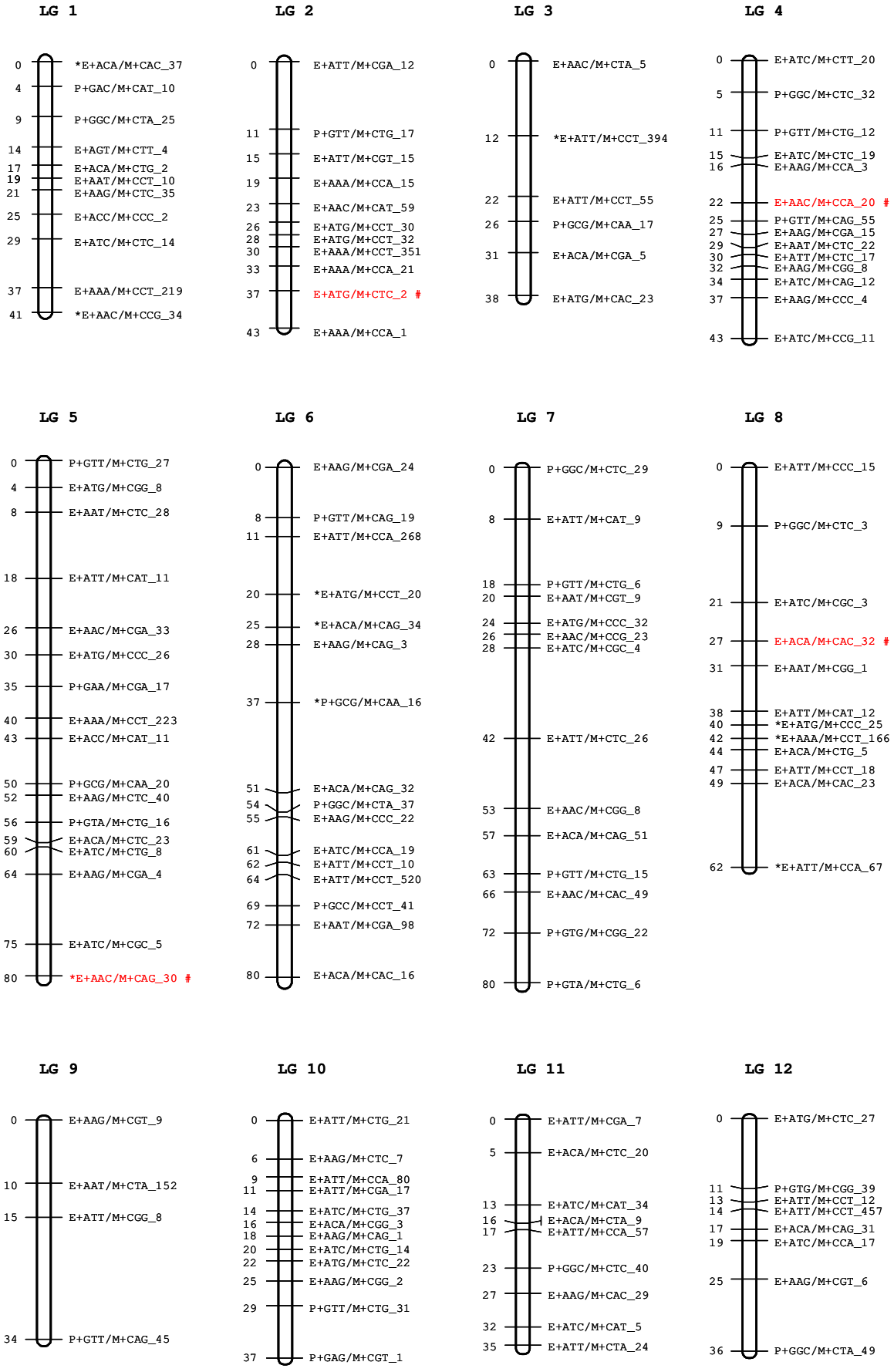


Fig. 26 continued

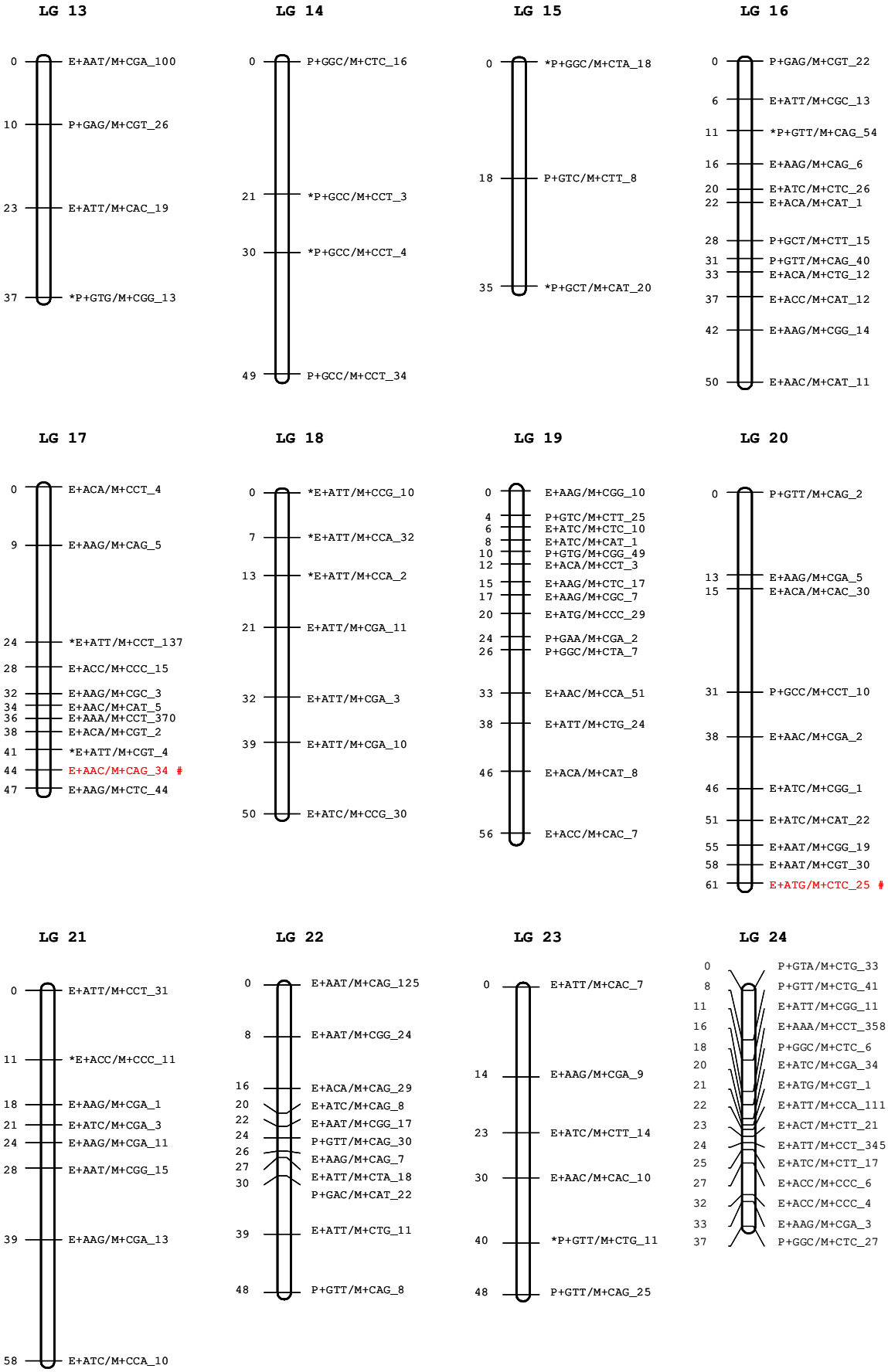


Fig. 26 continued

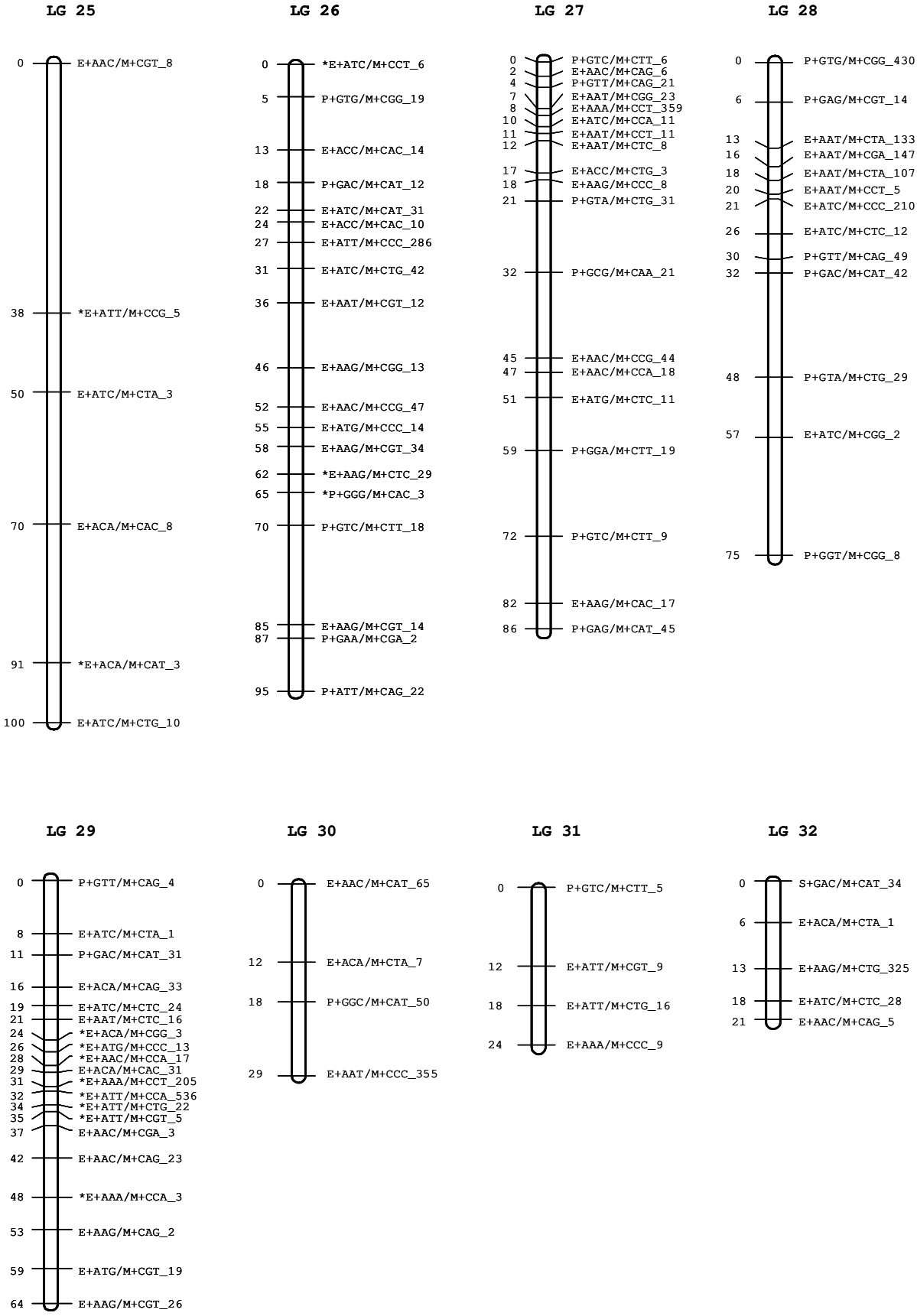


Fig. 26 continued

3.2.4 Marker alignment to the tetraploid population

Six hundred four scored markers from 13 selective primer combinations analyzed in the dihaploid “Artis” population were used for alignment studies. In total, 268 markers could be aligned to the tetraploid “Artis” population. Seventy-seven markers were from the female parent only and 191 markers were from both parents of the tetraploid population (Table 23).

Table 23: Allelic segregation in the tetraploid population with aligned markers from the dihaploid population

Alleles in dihaploids	**Alleles in tetraploids**							
Only from female parent	**Only from female parent (%)**				**from both parents (%)**			
Marker form	Simplex	skewed simplex	Duplex	skewed duplex	3 to 1	11 to 1	35 to 1	Mono-morphic
Simplex	30.4	7.0	1.2		34.6	20.0	2.9	3.9
Skewed simplex	30.1	6.0	3.0		41.9	10.0	3.0	6.0
Duplex	10.6	1.6	20.8	3.2	9.6	22.2	20.8	11.2
Skewed duplex	13.7	7.0	13.7		13.7	7.0	47.7	34.0
Tri- & Quaduplex			2.5	2.5			20.6	74.4

The aligned simplex markers showed simplex, skewed simplex and duplex constitutions in tetraploids if they were derived only from the female parent. Most of the aligned simplex (30.4 %) and skewed simplex (30.1 %) markers of the dihaploid population segregated again in a simplex or skewed simplex manner in the tetraploid population. Also 20.8 % of the duplex markers kept duplex constitution, but curiously several duplex markers were found to segregate in the tetraploid population as simplex or skewed simplex markers or vice versa.

With the allelic dosages derived from both parents, three theoretically different segregation types were found: 3 to 1 segregating ratio from simplex (♀) x simplex (♂) constitution, 11 to 1 from simplex (♀) x duplex (♂) or duplex (♀) x simplex (♂) and 35 to 1 from duplex (♀) x duplex (♂). However, 35 to 1 segregating ratios were also derived from simplex constitutions as well as 1 to 1 ratio.

3.2.5 Quantitative trait loci analysis

3.2.5.1 Chip color after long term storage at 4°C

Thirteen genetic markers explained over 10 % of phenotypic variance for chip color after long term storage at 4°C and ranged from 10.2 % to 24.9 %. They represented in total ten QTL in interval mapping (Table 24).

Table 24: Results of GLM and interval mapping of chip color after long term storage at 4°C

Molecular Markers			[1]GLM	Interval mapping		
Linkage group		Linked marker	F value	Area (cM)	LOD	R^2 %
I	(LG 5)	E+AAC/M+CAG_30	26.907	79.7-80.2	5.17	24.9
		E+ACC/M+CAT_11	9.416	35.0-42.8	1.98	10.4
I	(LG 17)	E+ATT/M+CCT_137	13.217	13.6-28.1	2.72	16.4
IV	A1	E+ATT/M+CCT_8	9.970	7.5-24.6	2.09	11.0
VII	A1	E+ACA/M+CTC_2	10.412	56.1-71.1	2.29	13.4
VII	A2	E+ATT/M+CTG_20	14.350	43.8-44.4	3.00	16.8
		E+ACC/M+CCC_8	10.866	13.6-35.8	2.27	11.8
IX	A1	E+AAC/M+CGT_11	10.309	36.8-53.3	2.16	11.3
XI	A1	E+AAA/M+CCC_5	9.193	5.0-24.1	1.94	10.2
XII	(LG 2)	E+AAA/M+CCA_15	10.662	14.7-18.8	2.23	11.6
	LG 3	E+ATG/M+CAC_23	9.420	36.2-38.4	1.98	10.4
	LG 19	E+ACA/M+CCT_3	10.412	10.2-16.6	2.18	11.4
		P+GGC/M+CTA_7	9.319	23.9-33.4	1.96	10.3

[1] **GLM** : The General Linear Models Procedure in **SAS**

Two QTL were mapped on chromosome I and VII, further QTL were mapped on chromosomes IV, IX, XI, XII, LG 3 and LG 19. In addition nineteen QTL for chip color explained 5 % to 10 % of phenotypic variance. Through automatic cofactor selection, three were set for cofactor in MQM analysis and mapped on chromosomes I, XI (A1) and LG 9. Five QTL explained together 58.1 % of phenotypic variance of chip color after long term cold storage at 4°C with a significance level of $p<0.0015$. (Table 25).

Table 25: MQM analysis of chip color after long term storage at 4°C

Molecular marker			MQM analysis			
Linkage group		Linked marker	Area (cM)	LOD	Partial R^2 %	R^2 %
I	(LG 5)	E+AAC/M+CAG_30	80.2	5.17	24.9	
VII	A1	E+ACA/M+CTC_2	59.3	2.18	11.4	
XI	A1	E+AAA/M+CCC_5	13.1	1.94	10.2	**58.10 %**
IV	A1	E+ATT/M+CCT_8	14.2	2.09	11.0	
I	(LG 17)	E+ATT/M+CCT_137	24.3	2.72	14.0	

Two QTL mapped on chromosome I and three QTL mapped on chromosome IV, VII and XI. According to various chip color evaluation data in regression analysis, these five QTL explained from 36.43 % to 58.1 % of the phenotypic variance as presented in Table 26 together with the additive effects.

Table 26: Additive effects of five QTL for chip color and total explained phenotypic variance according to different phenotypic evaluations

QTL			Chip color evaluations					
			1998	1999	2000	2001		Mean
Linkage group		Linked marker				Schlüter	Pulling	
			Additive effect (Chip color (1-9: light-dark))					
		Intercept	5.3	4.1	5.5	4.4	5.2	4.9
I	(LG 5)	E+AAC/M+CAG_30	1.3	2.5	0.9	1.4	2.1	1.6
VII	A1	E+ACA/M+CTC_2	-0.8	-1.0	-1.1	-1.2	-1.3	-1.2
XI	A1	E+AAA/M+CCC_5	-1.5	-0.9	-1.0	0.8	-0.9	-1.1
IV	A1	E+ATT/M+CCT_8	0.7	1.1	1.3	0.5	1.2	1.0
I	(LG 17)	E+ATT/M+CCT_137	-1.6	-1.2	-1.5	1.2	-0.8	-1.0
			Explained phenotypic variation (R^2)					
I	(LG 5)	E+AAC/M+CAG_30	11.5	32.0	8.3	16.7	29.3	24.9
VII	A1	E+ACA/M+CTC_2		5.5	9.8	11.3	8.7	11.4
XI	A1	E+AAA/M+CCC_5	8.5	6.2	6.7	5.2	8.6	10.2
IV	A1	E+ATT/M+CCT_8	5.2	9.3	11.6	3.5	7.8	11.0
I	(LG 17)	E+ATT/M+CCT_137	12.4	11.7	19.2	14.6	12.1	14.0
		Total (%)	32.2	52.7	42.5	43.5	52.6	58.1

One QTL on chromosome I (LG 5) was detected as main QTL among five QTL by explaining approximately 8.3 % to 32 %.

3.2.5.2 Glucose content after long term storage at 4°C

Based on interval mapping with the MapQTL program, 13 genetic markers explained over 10 % of phenotypic variance of glucose contents after long term storage at 4°C which represents a total of six QTL located on chromosomes I (LG 5, LG17), chromosome IX, X, XII (LG 2) and LG 10 (Table 27).

Table 27: Results of interval mapping for glucose content after long term storage at 4°C

Molecular Markers			[1]GLM	Interval mapping		
Linkage group		Linked marker	F value	area (cM)	LOD	R^2 %
I	(LG 5)	E+ATC/M+CGC_5	7.905	69.2-80.2	2.09	18.6
I	(LG 17)	E+ACA/M+CCT_4	11.740	0.0- 5.0	2.44	12.7
IX	A3	E+ATG/M+CCC_35	11.626	5.0- 6.1	2.42	12.5
X	A3	E+ACC/M+CCC_3	6.237	5.0-16.0	1.68	11.7
XII	(LG 2)	E+AAA/M+CCA_15	16.014	18.8-32.6	3.25	16.5
	LG 10	P+GAG/M+CGT_1	11.963	34.4-36.8	2.48	14.4

[1] **GLM** : The General Linear Models Procedure in **SAS**

The best QTL for glucose content was localized on chromosome XII (LG 2) explaining 16.5% of phenotypic variance with 3.25 of LOD value. Two QTL mapped on chromosomes I (LG 5) and XII (LG 2) were distinguished within a wide range. Further 9 genetic markers explain approximately 7 to nearly 10 % of phenotypic variance of glucose content and these scatter over chromosomes I, IV, V, VII, IX and XI. Two cofactors were selected in MQM and mapped on chromosomes IV (A1) and IX (A1). Five QTL located on chromosomes XII, IV, I, VII and LG10 explain together 48.73 % (Table 28) of phenotypic variance for glucose content after long term storage at 4°C with a significance level of $p<0.0001$.

Table 28: MQM analysis of glucose content after long term storage at 4°C

Molecular marker			**MQM analysis**			
Linkage group		Linked marker	Area (cM)	LOD	Partial R^2 %	R^2 %
XII	(LG 2)	E+AAA/M+CCA_15	18.8-27.7	3.94	16.9	**48.73 %**
IV	A1	E+ATT/M+CCT_8	14.2	1.59	8.0	
I	(LG 17)	E+ACA/M+CCT_4	0.0-18.6	3.34	14.6	
VII	A2	E+AAC/M+CGA_7	27.1-33.8	2.11	9.5	
	LG 10	P+GAG/M+CGT_1	34.4-36.8	2.46	12.3	

3.2.5.3 Fructose content after long term storage at 4°C

For fructose content, 10 genetic markers were detected explaining above 10 % of phenotypic variance in a total of eight QTL located on chromosomes I, VII, IX, X, XII and LG 10 (Table 29).

Table 29: Results of interval mapping for fructose content after long term storage at 4°C

Molecular Markers			[1]**GLM**	**Interval mapping**		
Linkage group		Linked marker	F value	Area (cM)	LOD	R^2 %
I	(LG 5)	E+AAC/M+CAG_30	9.773	69.2-80.2	2.73	20.2
I	(LG 17)	E+ACA/M+CCT_4	12.110	0.0- 8.6	2.51	13.6
		E+ATT/M+CCT_137	13.753	13.6-24.3	2.83	16.2
VII	A2	E+ATT/M+CTG_20	14.099	8.6-49.1	2.89	15.8
IX	A3	E+ATG/M+CCC_35	11.507	5.0- 6.1	2.39	12.4
X	A3	E+AAC/M+CAT_1	7.444	0.0-32.3	1.73	12.3
XII	A2	E+AAG/M+CCC_35	7.259	0.0-12.8	1.77	12.2
XII	(LG 2)	E+AAA/M+CCA_15	18.099	18.8-23.0	3.63	18.3
		E+ATG/M+CCT_32	11.849	27.7-32.6	2.46	12.8
	LG 10	P+GAG/M+CGT_1	12.014	34.4-36.8	2.49	15.5

[1] **GLM**: The General Linear Models Procedure in **SAS**

Nineteen genetic markers explained approximately 7 to nearly 10 % of phenotypic variance for fructose content and these scatter over 16 linkage groups. Three cofactors were selected by MQM analysis and mapped on chromosomes IV (A1) and IX (A1, A3). Eight QTL for fructose content explain together 66.74 % of phenotypic variance (Table 30). Five QTL for fructose contents were mapped on the same positions as the QTL for glucose content, however, the phenotypic variance R^2 was higher than for glucose content. They explain 49.89 % of phenotypic variance for fructose content.

Table 30: MQM analysis of fructose content after long term storage at 4°C

Molecular marker			**MQM analysis**			
Linkage group		Linked marker	Area (cM)	LOD	Partial R^2 %	R^2 %
XII	(LG 2)	E+AAA/M+CCA_15	18.8–29.6	2.91	10.0	
VII	A2	E+AAC/M+CGA_7	13.6–33.8	2.35	8.1	
I	(LG 17)	E+ACA/M+CCT_4	0.0– 5.0	1.98	8.0	
IV	A1	E+ATT/M+CCT_8	14.2	1.93	7.5	**66.74 %**
	LG 10	P+GAG/M+CGT_1	34.4–36.8	2.84	10.6	
IX	A1	E+ATC/M+CCG_12	17.0	3.47	14.2	
IX	A3	E+ATG/M+CCC_35	5.0– 6.1	4.95	21.1	
	LG 25	E+AAC/M+CGT_8	0.0–20.0	3.11	10.6	

3.2.5.4 Starch content

With interval mapping, 9 genetic markers that represent in total eight QTL for starch content after harvest were identified and mapped on chromosomes I, III, VI, VII, IX, LG 9 and LG 19. The explained phenotypic variance R^2 ranged from 10.0 % to 16.7 % (Table 31).

Table 31: Results of interval mapping of starch content after harvest

Molecular Markers			[1]**GLM**	**Interval mapping**		
Linkage group		Linked marker	F value	Area (cM)	LOD	R^2 %
I	(LG 5)	E+AAA/M+CCT_223	16.257	35.0–39.5	3.30	16.7
I	(LG 17)	E+AAC/M+CAG_34	10.075	31.6–47.4	2.12	11.1
III	A2	P+GTC/M+CTT_13	13.882	24.2–29.7	2.85	14.6
VI	A2	E+ATT/M+CGA_25	10.649	14.9–25.6	2.22	11.6
VII	A1	E+ATC/M+CCG_3	9.280	21.1–51.1	1.97	10.3
IX	A3	E+ATG/M+CCC_35	9.001	0.0– 6.1	1.91	10.0
		E+AAC/M+CCG_29	13.036	20.0–38.1	2.69	13.9
	LG 9	E+AAT/M+CTA_152	14.727	5.0–25.0	2.85	15.0
	LG 19	E+ATG/M+CCC_29	9.401	16.6–20.0	1.96	10.3

[1] **GLM**: The General Linear Models Procedure in **SAS**

In addition, there were eleven QTL explaining 7 to 10 % of phenotypic variance in interval mapping. Three were selected as cofactors in MQM and mapped on chromosomes I, IX and LG 9. Three QTL for starch content explained together 42.83 % (Table 32) of phenotypic variance. The three QTL were mapped to chromosome I, IX and LG 9.

Table 32: MQM analysis for starch content after harvest

Molecular marker			**MQM analysis**			
Linkage group		Linked marker	Area (cM)	LOD	Partial R^2 %	R^2 %
	LG 9	E+AAT/M+CTA_152	5.0–25.0	3.79	13.6	
I	(LG 5)	E+AAA/M+CCT_223	35.0–39.5	4.76	17.2	**42.83 %**
IX	A3	E+AAG/M+CCC_17	0.0– 6.1	3.38	11.7	

3.2.5.5 Comparison of QTL for chip color and reducing sugar content after long term storage at 4°C

Based on the previous results of MQM analysis, selected QTL for each trait were applied to the others traits. The phenotypic variances are summarized in Table 33.

Table 33: Explained phenotypic variances for chip colors and reducing sugar content after long term storage due to selected QTL from MQM

QTL				**QTL selected from**					
Chromosome		Linked marker	Code[A]	**Chip color**		**Glucose**		**Fructose**	
XII	LG 2	E+AAA/M+CCA_15	**A**			x		x	
VII	A2	E+AAC/M+CGA_7	**B1**			x		x	
VII	A1	E+ACA/M+CTC_2	**B2**	x					
IV	A1	E+ATT/M+CCT_8	**C**	x		x		x	
I	LG 17	E+ACA/M+CCT_4	**D1**			x		x	
I	LG 17	E+ATT/M+CCT_137	**D2**	x					
I	LG 5	E+AAC/M+CAG_30	**D3**	x					
XI	A1	E+AAA/M+CCC_5	**E**	x					
	LG 10	P+GAG/M+CGT_1	**F**			x		x	
IX	A1	E+ATC/M+CCG_12	**G1**					x	
IX	A3	E+ATG/M+CCC_35	**G2**					x	
	LG 25	E+AAC/M+CGT_8	**H**						
				glucose	fructose	chip	fructose	chip	glucose
Explained phenotypic variance (R^2)				27.1 %	27.3 %	**43.9 %**	49.1 %	**49.9 %**	60.9 %

[A] each QTL was randomly coded. “x” indicates the selection of QTL

The selected five QTL from MQM for chip color explained in total 58.1 % phenotypic variance, however, these QTL represent only approximately 27 % for glucose and fructose content. On the other hand, five QTL for glucose content explaining 48.7 % in MQM represented 43.9 % of phenotypic variance for chip color and 49.1 % for fructose content. Eight QTL for fructose content explained 49.9 % phenotypic variance for chip color and 60.9 % for glucose content. One QTL was detected as a common QTL for all traits on chromosome IV. All five QTL for glucose content are also QTL for fructose content. The position of QTL for chip color, glucose and fructose content after long term storage at 4℃ and starch content after harvest are presented in Fig. 27.

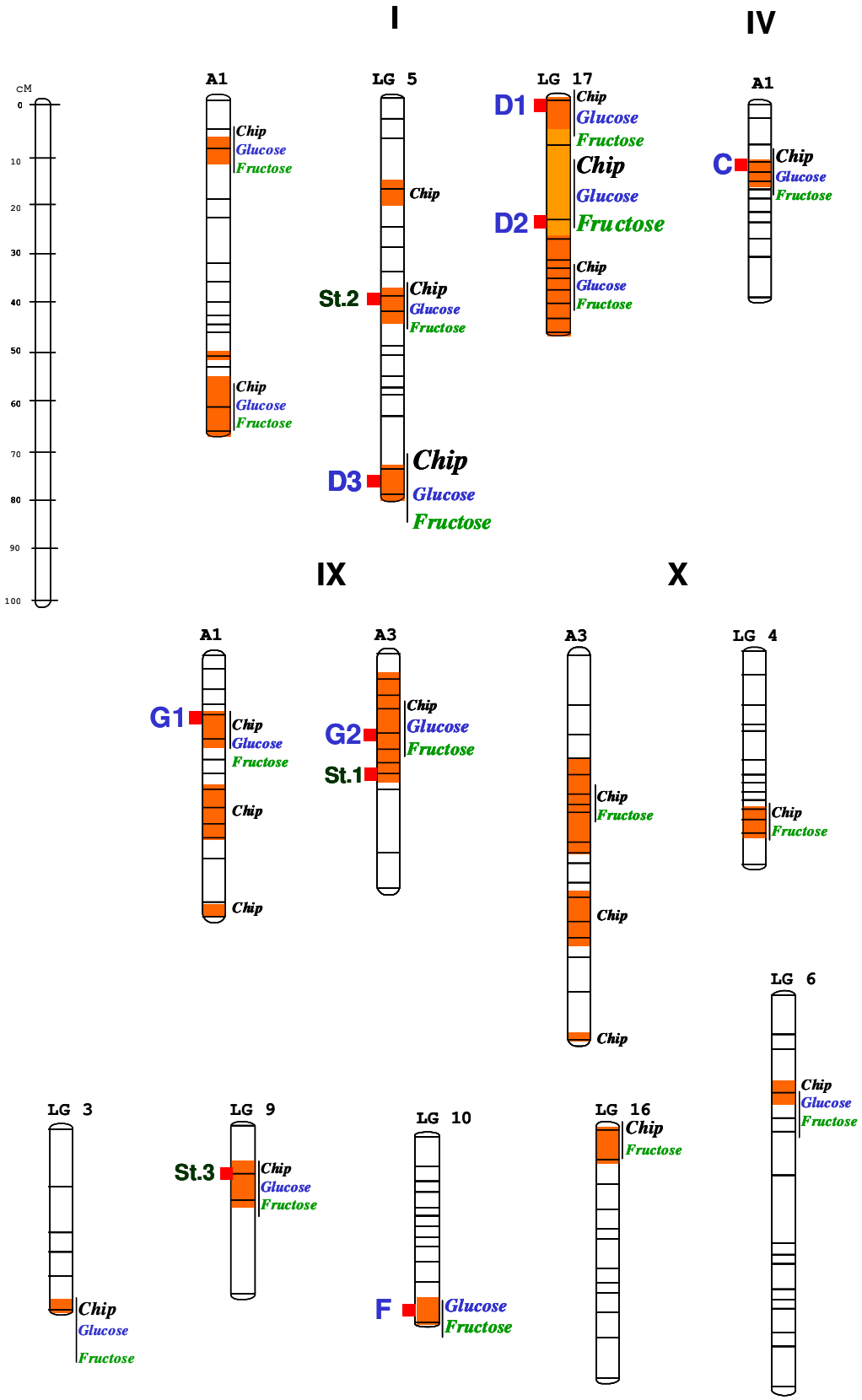


Fig. 27: Localization of QTL for chip color, glucose and fructose content after long term storage at 4°C and starch content after harvest in the dihaploid “Artis” population

A, B1, B2, C, D1, D2, D3, E, F, G1, G2 and H: QTL code according to table 33
St1, St2 and St3: QTL code for starch content

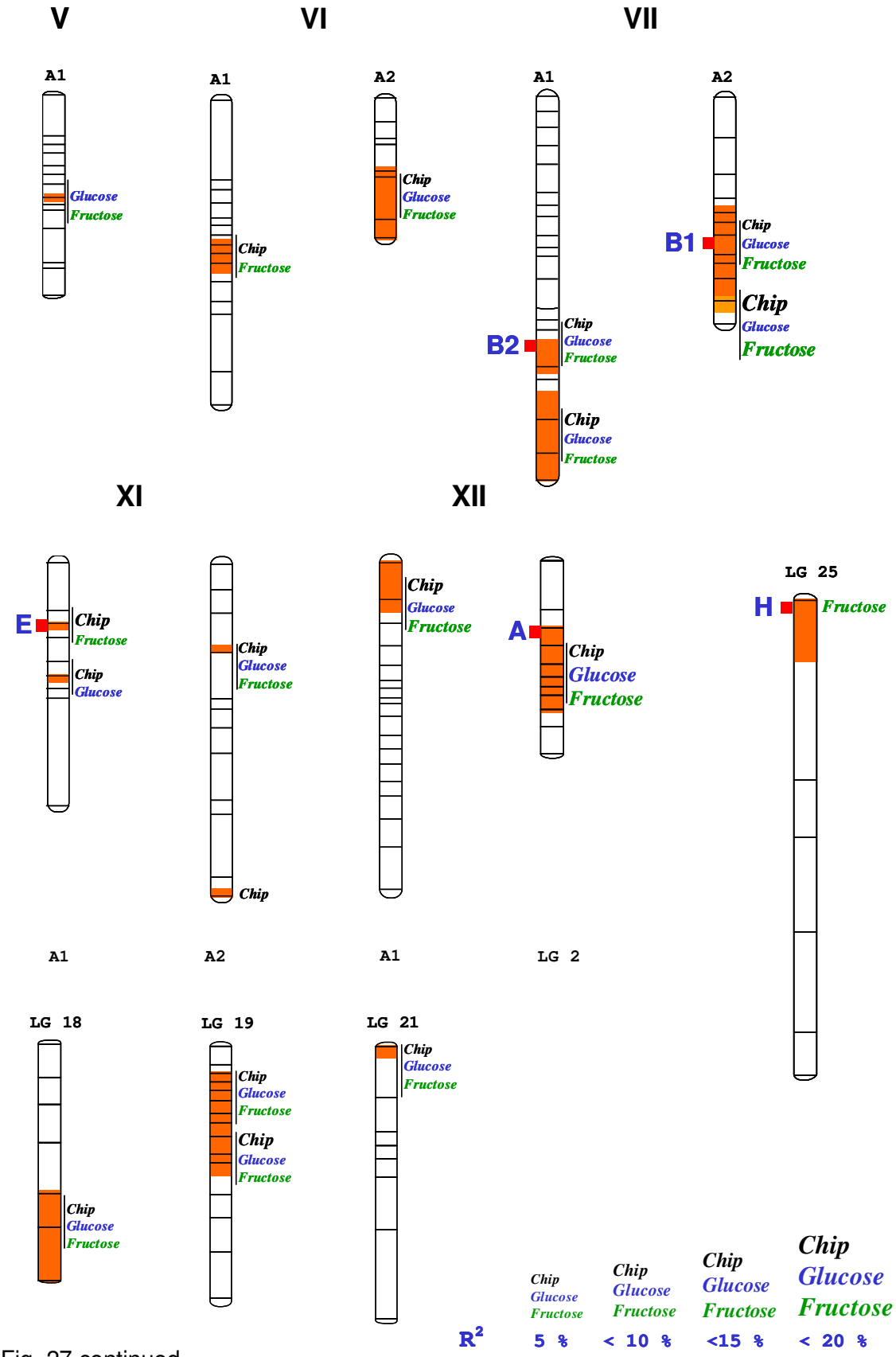


Fig. 27 continued

4 Discussion

4.1 Extreme resistance to PVY (*Ry$_{sto}$*)

4.1.1 Plant material

In this work, molecular markers were established by use of anther culture derived plants - a very unusual assay for marker development in potato. Unreduced tetraploids and mixploids were also found among the regenerated androgenic plants. Thirty-five (28.2 %) lines were tetraploids out of 124 anther culture regenerates that were derived from the cultivar “Assia”. 19 (15.3 %) lines were analyzed to have more than 24 chromosomes. Virus infection test started with 70 dihaploid lines, furthermore, 13 additional lines had to be removed from the population because they were detected as identical by AFLP analysis. These homologous copy lines were presumed from multi-embryo formation during anther culture. This process can occur if several plants were derived from the same individual anther and it was also observed in the other laboratories (Rokka, personal communication). Nevertheless, more regenerates from one anther should be taken because they also could be different as plants P76a-d demonstrate in this result.

Anther culture of potatoes is difficult and there are some limitations in obtaining large populations because of strong genotype specific influences of the donor plant on regeneration (Jacobsen & Sopory, 1978; Wenzel & Uhrig, 1981; Uhrig, 1985; Rokka *et al*., 1996; Schwarzfischer *et al*., 2002). In spite of occasional plant material problems, the use of anther culture derived plants was a very successful strategy for marker development.

4.1.2 Localization of the *Ry$_{sto}$* gene in the potato genome

According to Brigneti *et al*. (1997), the *Ry$_{sto}$* gene should be localized between the probes GP125 and CT182 on chromosome XI. The *Ry$_{adg}$* gene is also localized on the same position (Hämäläinen *et al*., 1997). PCR and SCAR markers were developed from the *Ry$_{adg}$* gene (Sorri *et al*., 1999; Kasai *et al*.,

2000) and used for marker-assisted selection (MAS) (Ruiz de Arcaute *et al*., 2002). We applied these markers in a spectrum of German potato cultivars but the results in 3.1.1 show that they are not suitable for cultivars having extreme resistance derived from *S. stoloniferum* (*Ry$_{sto}$*). This indicates that marker utility must be considered dependent on the genetic background of test material. Hämäläinen (1999) also failed to reproduce the *Ry$_{sto}$* localization on chromosome XI. Hämäläinen suggested that the PVY resistance genes of different species may be different or at different chromosomal loci. Also in practical potato breeding programs, the published *Ry$_{adg}$* markers are not suitable for breeding lines derived from *S. stoloniferum* (Ruiz de Arcaute *et al*., 2002). Celebi-Toprak *et al*., (2002) reported that no linkage was observed between molecular markers and the *Ny$_{tub}$* gene from chromosome XI and the *Ry$_{sto}$* locus. There was also no evidence that *Ry$_{adg}$* and *Ry$_{sto}$* represent different sources of allelic genes on distinct, linked loci. Together with our results, it seems that *Ry$_{sto}$* cannot be located on chromosome XI. Furthermore we found one SSR marker linked to chromosome XII co-segregating with *Ry$_{sto}$* markers indicating the localization of *Ry$_{sto}$* on this chromosome.

4.1.3 PCR marker development

AFLP marker analysis in dihaploid potato populations produces so many polymorphisms, that it is difficult to elute the target band for PCR marker development. One AFLP fragment was eluted, cloned and the PCR markers were designed based on sequence data (results in 3.1.7). Nevertheless, the PCR marker also worked in tetraploid potatoes but the bands were weaker than in the dihaploid population. This fragment is close to other bands with a distance of only 1 to 4 bp, but the possibility of STS marker development from AFLP fragments in potatoes exists.

4.1.4 Application of *Ry$_{sto}$* markers

Several *Ry$_{sto}$* markers were applied to cultivars and breeding lines and delivered convincing results. Recombination events found in the “Assia” population and presumably in the cultivars “Alwara”, “Arosa”, “Meduza” and “Nimfy” were obtained with more distinct markers. For some markers it was difficult to score

the target band in tetraploid potatoes whereas, in comparison, the bands were very clear in AFLP-assays with diploid potatoes. The characters of heterozygous plants appear complex and with high polymorphism. This affirms the difficulties that occur in the application of genetic markers in tetraploid potatoes with MAS.

The *R*y$_{sto}$ gene confers broad-spectrum resistance to three potyviruses, namely potato viruses A, Y and V. According to Barker (1996; 1997) the *Ry$_{sto}$* is a single gene or a cluster of closely linked genes at one locus. In our results, several *Ry$_{sto}$* markers are linked closely together but recombination was also observed in the dihaploid "Assia" population. No PVA or PVV resistance tests were performed with this population, however, with such recombinants it would be possible to clear the question whether PVA or PVV resistance derived from *S. stoloniferum* are inherited by *Ry$_{sto}$* or by independent clustered genes.

4.1.5 Extreme resistance (*Ry$_{sto}$*) and cytoplasmic male sterility

In practical potato breeding programs, the detected 19 cultivars with extreme resistance to PVY have been successfully crossed only as female parents due to cytoplasmic male sterility. Birhman & Kaul (1989) reported that cytoplasmic male sterility in potatoes ranges from partial to complete and is conditioned by the interaction of nuclear (*Fr*) and cytoplasmic (*c*) genes. All 19 cultivars mentioned above have specific mitochondrias classified as γ type (Table 17). This mitochondrial type correlates cytoplasmic male sterility (Lössl *et al*., 1999; 2000). Ross (1986) reported that PVY-immune *S. stoloniferum* back cross hybrids usually function only as females, however, exceptions are found yielding male fertile progenies. On the other hand, we analyzed the cytoplasm type of 10 different *S. stoloniferum* lines from the German potato gene bank by PCR markers and found only α type mitochondrias. These results agree to the mitochondria cluster analysis in the wide types (Lössl *et al*., 1999).

Recently it was possible to break the linkage of extreme resistance to PVY (*Ry$_{sto}$*) and cytoplasmic male sterility by protoplast fusion. In the protoplast fusion combination between a resistant dihaploid genotype to PVY with γ type mitochondria and a susceptible genotype with α type mitochondria, male fertile

somatic hybrids with α type mitochondria and extreme resistance to PVY could be regenerated (Schwarzfischer, personal communication). The plant material used in this study was derived from anther culture of the male sterile donor cultivar “Assia”. This circumstance shows that it is possible to regenerate androgenic plants from male sterile donor plants. Male sterility does not correlate with the formation of 2n microspores through the meiotic phase within the anther. This was indirectly proved by getting the androgenic dihaploids from male sterile donor cultivars, for example the dihaploids of cv. “Assia” in this study. A few diploids were also regenerated from male sterile cultivars “Petra” and “Ute” (unpublished data). Rokka (1998) also induced dihaploids from the male sterile cultivar “Petra” by means of anther culture.

4.1.6 Pedigree analysis

All cultivars and breeding lines identified with our Ry_{sto} markers are extreme resistant to PVY and descendents from MPI breeding lines with an *S. stoloniferum* genetic background. All extreme resistant cultivars from Germany belong to Group 1 or Group 3, which are derived from different cross parents. In the Polish cultivars, the extreme resistance to PVY was derived from breeding line MPI 47.174/3 which belongs to Group 1, in case of “Meduza” and “Klepa” whereas the cultivars “Nimfy” and “Hinga” belong to Group 2, a relatively small group with only Polish breeding lines and cultivars. The resistance level of Polish cultivars was kindly confirmed by Dr. Zimnoch-Guzowska at the Plant Breeding and Aacclimatization Institute (IHAR) in Poland. However, it is not clear whether the Ry_{sto} origin from the ancestors of the three groups in this pedigree analysis is identical.

As described in materials and methods, “Assia” was registered with extreme resistance to PVY and PVX. According to Ritter *et al*. (1991), there are two different resistance genes to Potato Virus X (*Rx*1, *Rx*2). *Rx*1 is localized on chromosome XII and derived from breeding line CPC1673 while *Rx*2 is localized on chromosome V and derived from breeding line MPI 44.1016/10. In the case of “Assia”, breeding line MPI 44.1016/10 was crossed as male to MPI 50.140/5 as female in the breeding pedigree so inherent “Assia” has the *Rx*2 gene, which is derived from *S. acaule.*

Cultivar “Fox” has high resistance level to potato virus Y and is derived from the same cross parents as “Assia”. However, the markers indicate that “Fox” is not extreme resistant to PVY (Ry_{sto}). The loss of the extreme resistance of “Fox” was personally confirmed by the potato breeder W. Zimmermann at the breeding station Uniplanta-Saatzucht KG.

Ross reported in 1986 that approximately 20 cultivars from the Netherlands, Poland, Hungary and Germany with *Ry* genes for extreme resistance to PVY and PVA bred as third to seventh backcross generation from *S. stoloniferum*. Seventeen among these 20 cultivars (1986) are summarized in Table 34.

Table 34: *Ry* gene origin of 17 European cultivated potatoes according to Ross (1986)

Cultivar	Registered year	[1]Nation	Origin of *Ry* gene		Pedigree group
			In the cross	Breeding line	
Corine	1976	NL	Male	Y 193 (CPC2093)	
Santé	1983	NL	Female, Male	Y 66-13-636, Y189 (CPC2093)	
Bóbr	1983	Po	Female	MPI 55.957/24	Group 3
Bzura	1983	Po	Female	PG 232	Group 3
Pilica	1983	Po	Female	MPI 55.957/24	Group 3
San	1980	Po	Female	MPI 55.957/54	Group 3
Magyar Rozsa	1980	H	Female	N-71	
Szignal	N.I.	H	N.I.	N.I.	
Barbara	1982	D	Female	MPI 64.956/68	Group 3
Bison	1968	D	Female	MPI 49.767/7	Group 1
Cordia	1974	D	Female	MPI 61.303/34	Group 1
Esta	1978	D	Female	MPI 61.303/34	Group 1
Fanal	1968	D	Female	MPI 54.4129/288	Group 1
Franzi	1977	D	Female	MPI 61.303/34	Group 1
Heidrun	1978	D	Female	MPI 61.303/34	Group 1
Pirola	1976	D	Female	MPI 185/14	Group 1
Wega	1978	D	Female	MPI 61.303/34	Group 1

N.I. means no information
[1] indicates registered nationality: NL (The Netherlands), Po (Poland), H (Hungary), D (Germany)

The pedigree of 13 cultivars coincided with the previous pedigrees in Fig. 18 based on breeding information and they are marked in blue in Fig. 28. Cultivar “Magyar Rozsa” is derived from the cross between N-71 and *tbr* according to Świeżyński *et al*. (1997). The breeding line N-71 (*sto* x *tbr* x *acl* x *tbr*) x (*dms* x *tbr*) has *S. stoloniferum* in the genetic background, but no further information was found. It was not possible to get pedigree information from the Hungarian cultivar “Szignal”. The Ry_{sto} gene origins of the cultivar potatoes “Corine” and “Santé” were derived from male parents, Y 193 and Y 189, descendants from *S. stoloniferum* CPC 2093.

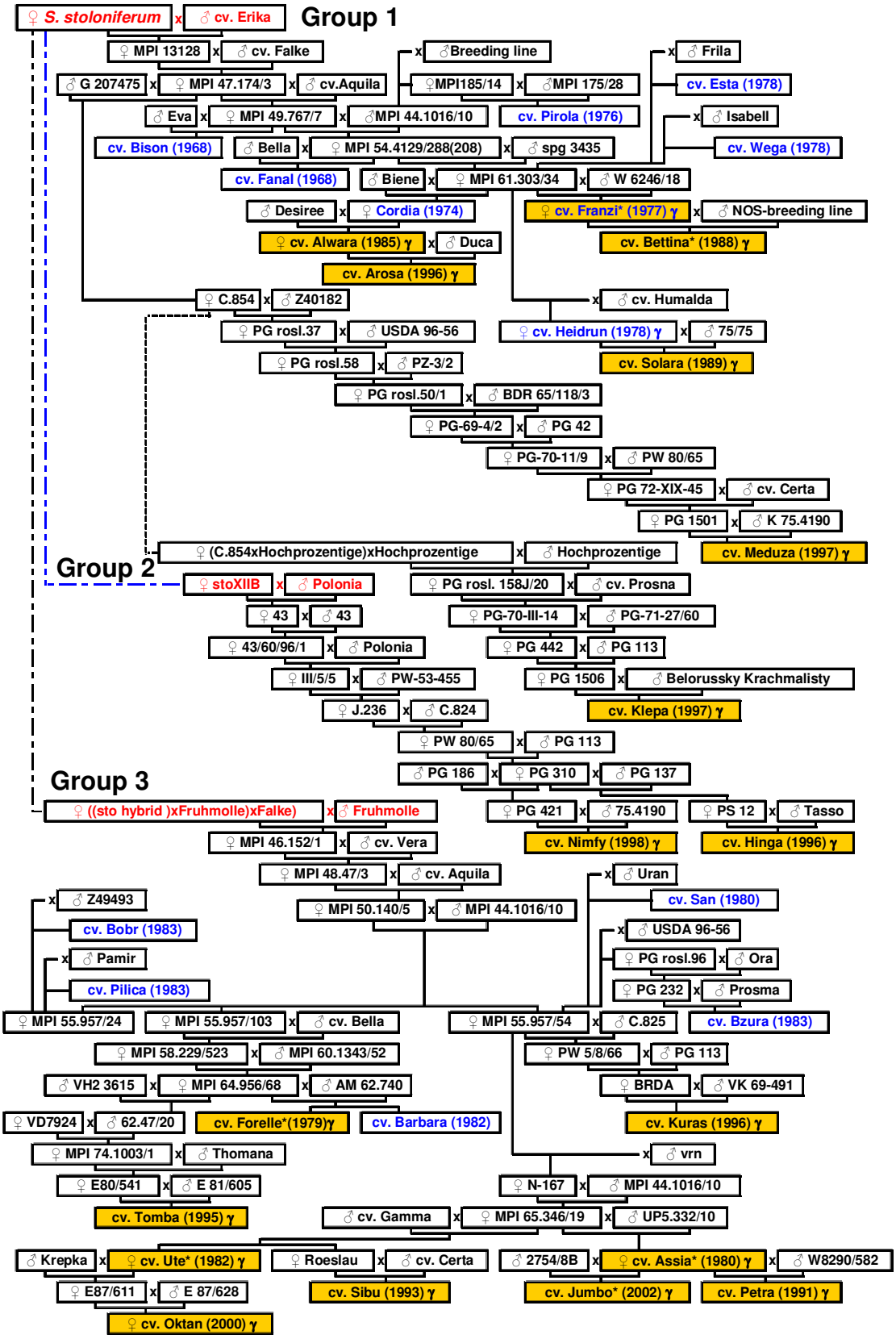


Fig. 28: Maternal pedigree of extreme resistant cultivars

The cultivars written with blue color indicate the extreme resistant cultivars listed by Ross (1986) and the cultivars in yellow boxes were detected to have the extreme resistance by marker selection.

Interestingly, CPC 2093 belongs to phenotype 3 according to Cockerham (1970) who classified 7 phenotypic reactions responding to PVY and PVA that was described already in the introduction part. This coincides with Barker (1996, 1997) who reported that the cultivar "Corine" carries the gene Ry_{sto}^{n1} (Ry_{sto}^{na}), which inherits resistance only to PVY but not to PVA. It can be assumed that "Corine" is not an extreme resistant cultivar, but a cultivar having a high resistance level to PVY. Bakker (1997) reported that the resistance gene Ry_{adg} is better than Ry_{sto} because plants with Ry_{sto} can indeed demonstrate negative results in ELISA, but show mild necrosis symptoms in some leaves and the stem. Nevertheless, most of the extreme resistant cultivars are derived from *S. stoloniferum.* From pedigree analysis of extreme resistant cultivars in this study it can be inferred that the MPI breeding lines with PVY resistance from *S. stoloniferum* developed by Ross (1954) were widely spread through Germany and whole Europe.

4.2 Chip color quality after long term storage at 4°C

4.2.1 Cold storage

Good chip quality after long term storage at 4°C is mainly determined by the amount of reducing sugars, especially fructose in tuber tissue. This conclusion of our results is in agreement with Menéndez *et al*. (2002). Storage was one very important factor in this study for obtaining good phenotypic evaluation data for chip color and reducing sugar content. Maturity of tubers, temperature, and the humidity of storage conditions can affect chip quality in addition to genetic factors.

4.2.2 Plant material

Population size and type are essential factors for the detection of QTL. Most mapping research for potatoes was constructed on the diploid level with inter-diploid populations with a size of 67 to 146 individuals (Bonierbale *et al*., 1988; Gebhardt *et al*., 1991; Van Eck *et al.,* 1995). Hackett *et al.* (1998) suggested in the simulation study that a population size of at least 150 individuals should be

used and that a larger number of 250 individuals would provide a better chance for identifying homologous chromosomes because selective genotyping of the population can decrease the amount of molecular analysis without great loss of detection power (Lander & Botstein, 1989).

In this study, for the first time a primary dihaploid population was used. For potatoes, it is very difficult to obtain a population with over 100 dihaploid individuals because many lines do not have sufficient vitality to produce enough harvest for a complete phenotypic evaluation. In here, only 83 individuals out of 337 dihaploid lines delivered the required phenotypic data. Wu *et al*. (1992) reported that a haploid population derived from a highly heterozygous polyploid plant would be the most efficient population in terms of maximization of Single-Dose Restriction Fragments (SDRF) detectability for mapping. On the other hand, tetraploid populations were favored for QTL analysis in potatoes during last years (Bradshaw *et al*., 1998; Meyer *et al*., 1998) and in autotetraploid sugarcane (Da Silva *et al.,* 1993, 1995) because detected QTL from diploids could often not be transferred to tetraploid cultivated potatoes in practical breeding programs. This study confirms the problem in marker alignment from the diploid to the tetraploid level (3.2.4).

4.2.3 Marker segregation and linkage group

In this study, simplex segregating markers composed approximately 52 % of all polymorphisms with differences due to restriction enzymes (Table 20), while 13.9 % of distorted simplex segregating markers were also found. These results are in agreement with the genetic study from Pande (2002). A distorted segregation is not logical, however, many potato genetic studies mentioned it (Gebhardt *et al*., 1991; Kreike, 1995; Van Eck, 1995). It could be considered that aberrant Mendelian segregation ratios may be the results of selection processes taking place during sporogenesis, gametogenesis, pollination, fertilization, seed development or germination (Van Eck, 1995). Gebhardt *et al*. (1991) found aberrant ratios with up to 40 % of the loci mapped on several chromosomes. In a primary dihaploid population of potatoes 48 linkage groups inherited from just one female parent can be expected in theory. The linkage groups were constructed like a F_1 backcross model with simplex segregating

markers even though dihaploid potatoes are composed of 24 chromosomes in cytology. In linkage analysis, 48 linkage groups (12 chromosomes x 4) were constructed in the JoinMap (Stam, 1993) program with an F_1 backcross model where one chromosome is represented by 4 linkage groups per chromosome derived from the genomes of the cultivar “Artis”. Alternatively TetraploidMap (Hackett & Luo, 2003) was applied to this dihaploid population (non published data), but it failed to analyze the linkage groups further because of the absence of sufficient co-dominant markers. Recently the Ultra High Density-map (http://www.dpw.wau.nl/uhd/) supports a lot of AFLP marker information for the potato and can help in marker alignment studies and for the attachment of linkage groups to specific chromosomes. According to the experience of this study, there are still limitations, however, in analyzing populations composed of specific genetic backgrounds.

4.2.4 QTL analysis

Interval mapping results delivered QTL with approximately 9 to 13 linked genetic markers scattered over a total of 26 linkage groups corresponding to chromosomes I, IV, V, VII, IX, X, XI and XII and explain each trait with more than 10 % of the phenotypic variance. MQM analysis results reduced the number to leave several main QTL. However, the LOD range in MQM from 1.59 to 5.17 was not high. The power of MQM was not effective in this population due to relatively high QTL scattering over whole linkage groups. The marker alignment from the diploid to the tetraploid population was tried but skewed segregations were often found in the tetraploids and so only a few markers could be aligned. The best QTL marker with the highest explained phenotypic variance on the dihaploid level was not the best by application in the tetraploid population. Reasons for this negative result might be heterozygous allelic effects (Thill & Peloquin, 1994) caused by the other crossing parent or the increasing of the ploidy level.

The mechanism and regulation of the biochemical processes leading to cold sweetening is in large parts still unclear, however, the previously published QTL correlated with the candidate gene approach based on metabolic data (Chen *et al.*, 2001; Menéndez *et al.*, 2002). In addition, the results of this study in QTL

analysis of dihaploids confirmed results from Menéndez *et al*. (2002) who reported QTL for glucose, fructose and sucrose content that were similarly spread over all potato chromosomes with two inter-diploid populations. The linkage groups of the dihaploid "Artis" population are composed of mostly anonymous AFLP markers and small numbers of SSR and RFLP markers. Therefore, the comparison with the results of Menéndez *et al*. (2002), summarized in Table 35, was helpful in estimating candidate genes.

Table 35: Comparison of QTL for reducing sugar content, chip color and starch content with published results

Menéndez *et al*., 2002						**This study (dihaploid "Artis" population)**					
Linkage groups	R^2(%)[1] in significant for traits[1]			QTL	candidate gene locus[3]	Linkage groups	QTL	R^2(%)[1] in significant for traits[1]			
	G	**F**	**S**					**G**	**F**	**C**	**St**
I	13.4	12.5		*Sug1a*	*AGPaseS*	**I**	**D1**	14.6	8.0		
							D2			14.0	
							D3			24.9	
							St2				17.2
						IV	**C**	8.0	7.5	11.0	
V		5.8	4.0	*Sug5a*	*Sut2*						
VII	5.1	5.0	4.2	*Sug7a*	*Sps*	**VII**	**B1**	9.5	8.1		
	15.1	13.3	12.0	*Sug7b*	*Sus3*		**B2**			11.4	
VIII	13.3	13.9		*Sug8b*	*AGPaseS*						
IX	7.5	14.5	5.7	*Sug9a*	*Inv-ap*	**IX**	**G1**		14.2		
							G2		21.1		
							St1				11.7
X	5.0	3.0		*Sug10a*	*Inv-ap*						
XI	7.5	15.0		*Sug11a*	*Sut1*	**XI**	**E**			10.2	
							F	12.3	10.6		
						XII	**A**	16.9	10.0		
						LG 25	**H**		10.5		
						LG 9	**St3**				13.6

[1] R^2 (%) is the value of phenotypic variance explanation, [2] Traits stands for "G" glucose, "F" fructose, "C" chip color and "St" starch content, [3] ADP-glucose pyrophosphorylase S and B (*AGPaseS* and *AGPase B*), Sucrose transporter 1 and a putative sucrose sensor (*Sut1* and *Sut2*), Cleaved amplified polymorphic sequence (CAPS) markers for sucrose phosphate synthase (*Sps)*and sucrose synthase 3 *(Sus3*; Chen *et al*., 2001), apolast invertase *(Inv-ap)*

Our results confirmed reference results of QTL for glucose content on chromosomes I, VII and XI. For fructose, QTL were mapped on chromosomes I, VII, IX and XI in both maps. QTL for fructose content were mapped on the same linkage group positions to which QTL for glucose content were positioned, in agreement with the results of correlation (3.2.1.2). QTL for chip color explained 27 % of the phenotypic variance of reducing sugar whereas QTL for reducing sugar explained 43.9 % to 49.9 % of the phenotypic variance for chip color. This indicates that there are other important factors for chip color besides the accumulation of reducing sugars. QTL explaining over 10 % of the variability for

chip color were located on chromosomes I, IV, VII, IX, XI and XII from interval mapping results. Chromosome IX is known to harbor the *invertase* gene (Salamini & Schneider, 2002; Menéndez *et al*., 2002) and UGPase activity (Sowokinos, 1990; Sowokinos *et al*., 1997), which closely related to glucose content under cold stress.

4.3 Potential of established markers for potato breeding

Genetic marker development in potatoes is very important for scientific studies, but also for practical utility in breeding programs. Marker assisted selection (MAS) is very attractive for the breeder, because it can facilitate and accelerate the selection of desirable genotypes. MAS can improve cost-effectiveness and significantly speed up the introgression of resistance genes into potato cultivars. In classical crossing schemes, 99% of the material is discarded within the first 3 years based on visual evaluation of foliage and tubers (Ross 1986; Tanksley *et al*., 1989; Watanabe *et al*., 1994). Until now, marker assisted selection (MAS) has been limited (Oberhagemann *et al*., 1999) or inefficient in potato breeding due to the lack of markers linked to useful traits in tuber-bearing S*olanum* including the cultivated potatoes.

In this study, an important breeding trait -extreme resistance to PVY (Ry_{sto})- was analyzed based on good phenotypic evaluation data involving marker development on the diploid level in order to establish MAS for diploid and tetraploid genotypes. Furthermore, these resistance markers were mapped on chromosome XII according to reference map (Milbourne *et al*., 1998) and the pedigree of extreme resistance to PVY (Ry_{sto}) in marker selected potato cultivars was confirmed. As extreme resistance to PVY (Ry_{sto}) is inherited by a single gene, this strategy of marker transfer from diploid to tetraploid level was proved successful and this good results indicate an optimistic possibility of the accession of MAS in breeding programs.

On the other hand, application of MAS is still difficult for qualitative traits, such as chip quality, starch content, nematode or late blight resistance. In this study

some QTL for good chip quality after long term storage at 4°C were developed for a dihaploid population and QTL analysis results confirm published regions for candidates genes. These could be used in further expression analysis studies and offer one more stepping-stone for the complex trait of cold sweetening.

5 Summary

This dissertation reports the development of genetic markers associated with extreme resistance to PVY (*Ry$_{sto}$*) and QTL referring to chip quality after long term storage at 4°C, a qualitative and a quantitatively inherited trait of the potato. The markers for extreme resistance to PVY were established within a primary dihaploid mapping population composed of 57 anther culture-derived lines from the cultivar "Assia" having *Ry* immunity from *S. stoloniferum* and *Rx* immunity. The genetic constitution of extreme resistance to PVY of "Assia" could be assumed as simplex (*Ryryryry*), which results in an approximate segregation of dihaploids ratio of 28 resistant to 29 susceptible (*Ry:ry*) lines. Twelve *Ry$_{sto}$* AFLP markers were selected out of 480 selective primer combinations in AFLP assays (*Pst*I/*Mse*I, *Eco*RI/*Mse*I) using Bulk Segregant Analysis. Nineteen potato cultivars ("Alwara", "Arosa", "Assia", "Bettina", "Dania", "Franzi", "Forelle", "Hinga", "Jumbo", "Klepa", "Kuras", "Meduza", "Nimfy", "Oktan", "Petra", "Sibu", "Solara", "Tomba" and "Ute") out of 110 tested potato cultivars from Germany, The Netherlands and Poland were detected by these markers as immune to PVY in alignment with very good phenotypic resistance data. According to linkage analysis, the *Ry$_{sto}$* resistance markers are not linked to chromosome XI in contrast to a previous publication (Brigenti *et al.*, 1997) but linked to chromosome XII based on the reference map of Milbourne *et al.* (1998). According to genetic pedigree analysis, these cultivars are derived from MPI breeding lines (Ross, 1986) or other sources with *S. stoloniferum* background. Thirteen immune potato cultivars listed by Ross were also recorded in this pedigree.

Chip color tests were performed from 1998 to 2001 with 83 dihaploid lines derived from the cultivar "Artis" having a good chip color after long term storage at 4°C. The chip color of the "Artis" dihaploid population showed continuous variation and correlated highly with glucose (r=-0.67) and fructose (r=-0.73) content. An F_1 backcross population mode was applied to construct 57 linkage groups covering 2085 cM. Twenty-five linkage groups covering 1379 cM could be assigned to a distinct chromosome number with 2342 AFLP, 33 SSR and 18 RFLP in a simplex manner. In addition 6 linkage groups covering 336 cM could

be identified by marker alignment with the Ultra High Density map. QTL analysis revealed that QTL for chip color, glucose and fructose after long term storage at 4°C scatter over 26 linkage groups. QTL explaining over 10 % of the variability for chip color, glucose and fructose content were located on chromosomes I, IV, VII, IX, XI and XII as a result of interval mapping. Five QTL mapped on chromosomes I, IV, VII and XI explained together 58.1 % of the phenotypic variance of chip color after long term cold storage at 4°C with a significance level of $p<0.0015$. All five QTL for glucose content mapped on the same positions as QTL for fructose content in agreement with the correlation results. Five QTL for glucose content explaining 48.7 % represented 43.9 % of the phenotypic variance for chip color and 49.1 % for fructose content. Eight QTL for fructose content explained 49.9 % of the phenotypic variance for chip color and 60.9 % for glucose content. In addition three QTL for starch content explained together 42.83 % of the phenotypic variance and were mapped to chromosomes I, IX and LG 9.

Our results in QTL analysis of dihaploids showed that the reducing sugar content contributes to the genetic variation of chip color after long term storage at 4°C. These results could be used in further expression analysis studies and offer one more stepping-stone for the complex trait of cold sweetening.

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