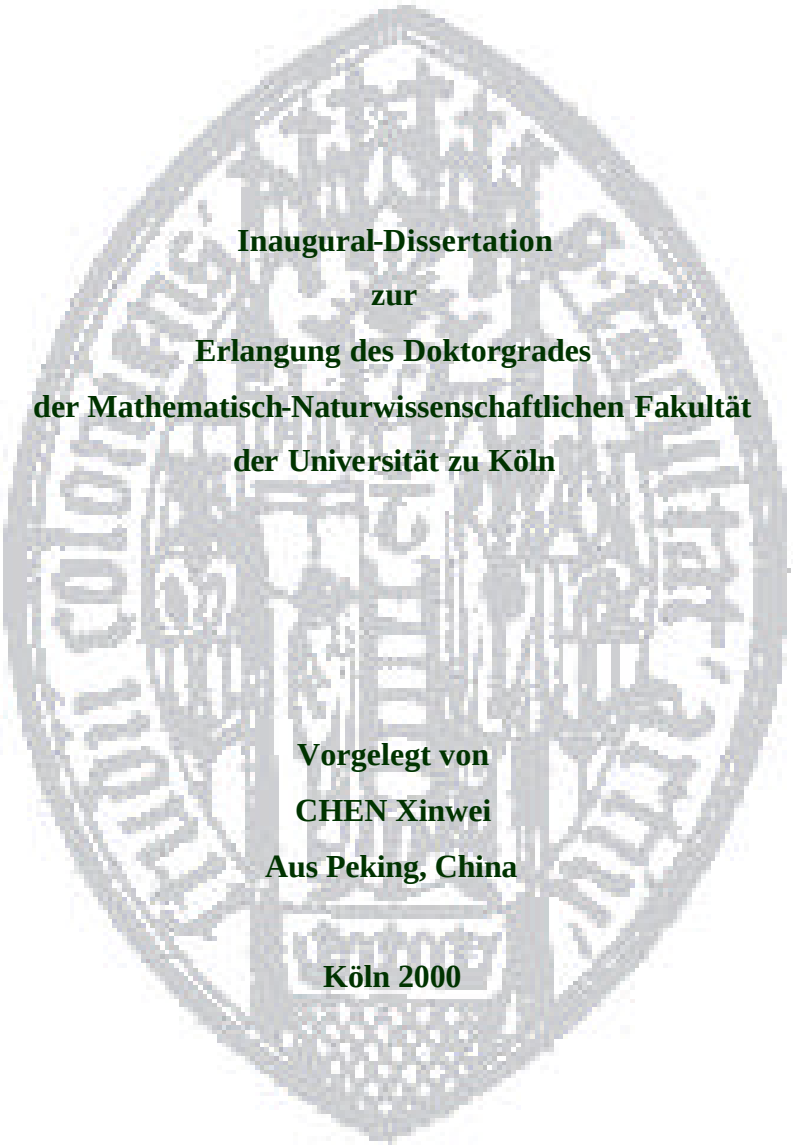
Molecular Mapping of Genes Involved in Carbohydrate Metabolism and Fluorescent AFLP-based Tagging of QTL in Tetraploid Potato



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for my father

Abbreviations

AFLP	amplified fragment length polymorphism
Amp	ampicillin
ANOVA	analysis of variance
APS	ammonium persulphate
bp	base pairs
BSA	bovine serum albumin / bulked segregant analysis
cDNA	complementary deoxyribonucleic acid
CAPS	cleaved amplified polymorphic sequence
cM	centiMorgans
dATP	deoxyadenosinetriphosphate
dCTP	deoxycytosinetriphosphate
dGTP	deoxyguanosinetriphosphate
dTTP	deoxythymidinetriphosphate
DH	doubled haploid
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylenediamine tetraacetic acid
EST	expressed sequence tag
EtBr	ethidium bromide
EtOH	ethanol
fAFLP	fluorescence-labelled AFLP
FE	foliage earliness
GLM	general linear models
h	hour(s)
IPTG	isopropylthio-β-o-galactopyranoside
kb	kilo base
LOD	logarithm of odds
M	Molar
MAS	marker-assisted selection
min	minute(s)
PCR	polymerase chain reaction
QTL	quantitative trait loci
rAFLP	radiolabelled AFLP
RAPD	randomly amplified polymorphic DNA
RFLP	restriction fragment length polymorphism
RIL	recombinant inbred line
RSC	reducing sugar content
s	second(s)
SCAR	sequence characterised amplified region
SDS	sodium dodecyl sulfate
SNP	single nucleotide polymorphism
STS	sequence tagged site
SSR	simple sequence repeat
SSCP	single-strand conformational polymorphism
SY	starch yield
TSC	tuber starch content
TY	tuber yield
X- gal	5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside

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1. INTRODUCTION

1.1 Taxonomy and importance of the potato

Cultivated potato (*Solanum tuberosum ssp. tuberosum*) belongs to the large and diverse genus *Solanum* of the family *Solanaceae*. *Solanaceae* is one of the largest and economically most important families of angiosperms. Besides potato, other economically important species of the family are tomato (*Lycopersicon esculentum*), egg-plant or aubergine (*Solamun melongena*), the garden peppers (*Capsicum annuum*) tobacco (*Nicotiana tabacum*) and pepino (*Solanum muricatum*). A number of ornamentals, petunia (*Petunia hybrida*) for example, also belong to the *Solanaceae*.

As a food crop, potato is one of the most important crops worldwide ranking 4th in terms of total world production after wheat, maize and rice. Potato tubers are valued for their high starch content. Starch can account for 80 % of the dry-weight of mature potato tubers (Kruger 1997), thus, it is an especially rich source of energy. Compared to cereals, potato yields on average more food energy and protein per unit of land (Horton 1988). Potato tubers also contain nutritive compounds such as high-quality protein (2 % of fresh weight) and substantial amounts of essential vitamins, minerals and trace elements. The production of protein per unit area of crop is second only to soybean (Tarn *et al*. 1992). The lysine content of potato can complement cereal-based diets that are deficient in this amino acid (Ortiz 1998). The importance of potato has been demonstrated by the devastating effect of the Irish famine in the 1840s. The continuing importance is reflected by the increasing growth rate of potato production, which has exceeded that of most other food crops in recent years in developing countries, particularly in Asia (Maldonado *et al*. 1998).

1.2 Evolution of cultivated potato

Potato is one of as many as 235 tuber-bearing *Solanum* species recognized to date, of which 228 are wild and 7 are cultivated (Hawkes 1990). Four wild species including *S. acaule, S. sparsipilum, S. leptophyes*, and *S. megistacrolobum* were believed to be involved in the evolution of the 7 cultivated species including *S. tuberosum* ssp. *andigena, S. tuberosum* ssp. *tuberosum, S. curtilobum, S. juzepczukii, S. stenotomum, S. chaucha, S. ajanhuiri,* and *S. phureja.* They occur in a range of chromosome numbers from 24 (diploid), 36 (triploid), 48

(tetraploid) to 60 (pentaploid). The evolutionary relationships are schematically presented in Figure 1-1.

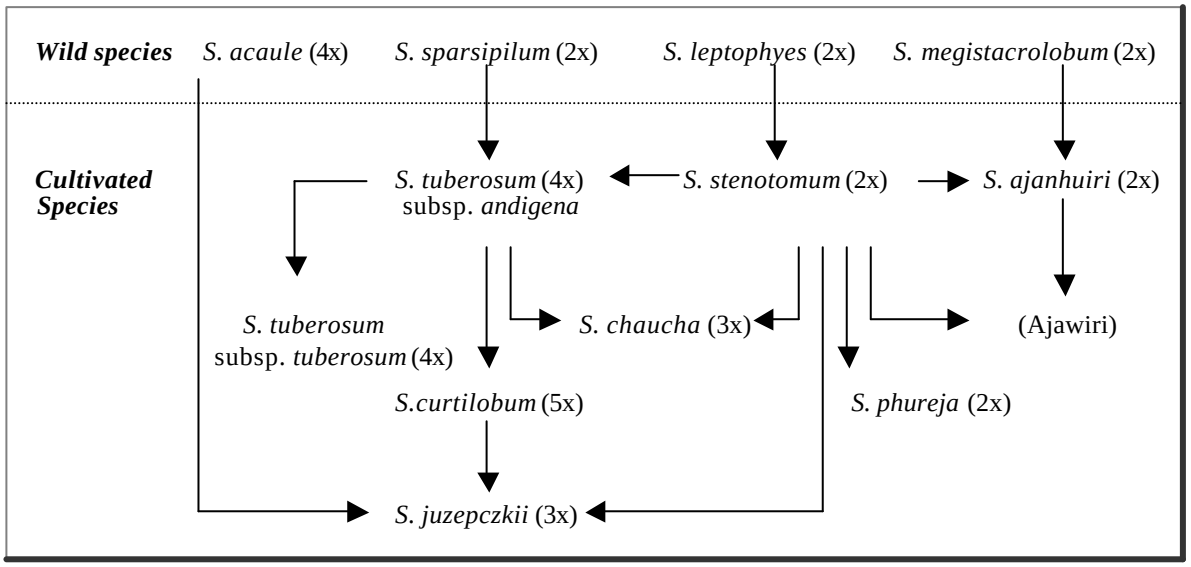


Figure 1-1 Evolutionary relationships of cultivated potatoes and their ploidy levels (adapted from Hawkes, 1990)

1.3 History of the cultivated potato

Potato is one of the most important world crops, yet until the 16th century it was restricted to South America and remained unknown to the people of Europe, Asia, Africa and North America. It is generally believed that potato was introduced to Europe as early as 1565 (Thornton, 1980) from Northern Colombia and further casually introduced in the 17th and 18th centuries (Bradshw and Mackay 1994). Potato was then introduced to China, India and Japan and part of Africa in the 17th century. The first European potatoes were short-day adapted tetraploid potatoes (*Solanum tuberosum* ssp. *andigena*) and restricted to the South of Spain and Italy (Hawkes, 1978). After a few centuries of natural and unconscious selection in Europe, plant could produce tubers under the long-day photoperiodic condition and became a popular crop in other parts of Europe. The cultivated potato in Europe today has the botanical name *Solanum tuberosum* ssp. *tuberosum*.

1.4 Genetic features of the potato

The cultivated potato is represented by both tetraploid and diploid forms with four and two homologous sets of 12 chromosomes, respectively. Most diploid potatoes (*S. tuberosum* ssp. *tuberosum*) exhibit self-incompatibility of the gametophytic type, in which the pollen self-incompatibility is determined by the haploid genotype of the pollen. This self-incompatibility is controlled by the single, multi-allelic locus S (De Nettancourt 1977, Kaufmann *et al.* 1991)

which was mapped on chromosome I (Gebhardt *et al*. 1991). Tetraploid potato and other polyploid species are generally self-compatible because of the action of more than one S allele in the pollen grain (Crane *et al*. 1929). They suffer, however, inbreeding depression after a few generations of selfing. Consequently, potatoes are highly outcrossing and highly heterozygous with multiple alleles at virtually every locus. This implies that random pairing

Table 1-1 Allele configurations in diploid and tetraploid potato and their progeny

A

? \ ?	***A1****	***A2***
A3	A1A3	A2A3
A4	A1A4	A2A4

*A1, A2, A3, …A8 are different alleles at one locus. For two given parental lines the number of segregating alleles could be as high as 4 and 8 for diploid and tetraploid plant, respectively. The possible genotypes of gametes (in bold italics) and zygotes are indicated as in **A** for diploid and **B** for tetraploid plants without considering chromatid segregation.

B

? \ ?	***A1A2***	***A3A4***	***A1A3***	***A2A4***	***A1A4***	***A2A3***
A5A6	A1A2A5A6	A3A4A5A6	A1A3A5A6	A2A4A5A6	A1A4A5A6	A2A3A5A6
A7A8	A1A2A7A8	A3A4A7A8	A1A3A7A8	A2A4A7A8	A1A4A7A8	A2A3A7A8
A5A7	A1A2A5A7	A3A4A5A7	A1A3A5A7	A2A4A5A7	A1A4A5A7	A2A3A5A7
A6A8	A1A2A6A8	A3A4A6A8	A1A3A6A8	A2A4A6A8	A1A4A6A8	A2A3A6A8
A5A8	A1A2A5A8	A3A4A5A8	A1A3A5A8	A2A4A5A8	A1A4A5A8	A2A3A5A8
A6A7	A1A2A6A7	A3A4A6A7	A1A3A6A7	A2A4A6A7	A1A4A6A7	A2A3A6A7

Table 1-2 Phenotypic segregation ratios of a dominant allele *A* for the crosses of tetraploid genotypes (without considering double reduction)

	Gametes ?			Gametes ?			Zygotes					PSR[b]
Type of crosses [a]	*AA*	*Aa*	*aa*	*AA*	*Aa*	*aa*	A^4	A^3a	A^2a^2	Aa^3	a^4	a:A
NxS a^4 x Aa^3	-	-	1	-	1	1	-	-	-	1	1	1:1
NxD a^4 x A^2a^2	-	-	1	1	4	1	-	-	1	4	1	1:5
SxS Aa^3 x Aa^3	-	1	1	-	1	1	-	-	1	2	1	1:3
SxD Aa^3 x A^2a^2	-	1	1	1	4	1	-	1	5	5	1	1:11
DxD A^2a^2 x A^2a^2	1	4	1	1	4	1	1	8	18	8	1	1:35

[a] N=Nulliplex, S=Simplex, D=Duplex.
Other types of crosses such as NxN, NxT (T=triplex), NxQ (Q=quadruplex), SxT, SxQ, DxT, DxQ, TxT, TxQ, QxQ, which do not segregate in the progeny, are not shown in the table.
[b] PSR= phenotypic segregation ratio.

of four homologous chromosomes at meiosis results in a large number of possible allelic combinations at a single locus. In the most extreme case of tetrasomic inheritance, eight different alleles descending from both heterozygous parents could segregate independently in a population, resulting in 36 possible genotypic classes in the progeny. The possible allelic configurations of both disomic and tetrasomic inheritance is shown in Table 1-1 (on page 3). The phenotypic segregation ratios are variable, ranging from 1:1 to 1:35, which depend on the dosage of parental alleles considered (Table 1-2, on page 3).

1.5 Molecular mapping in plants

1.5.1 Mapping populations

The construction of a linkage map is a process that follows the segregation of molecular markers in a segregating population and put them in linear order based on pair wise recombination frequencies . Thus, a mapping population with high number of polymorphisms over the total genome is highly desirable. Towards this end, various ways have been used to create mapping populations which are illustrated in Figure 1-2 (on page 5). Populations used for mapping are usually derived from F1 hybrids between two lines (either homozygous or heterozygous) which show allelic differences for selected probes. Specific populations can be further developed by various ways depending on the genetic features of the plant species being analysed. The commonly used populations are doubled haploid (DH) lines, recombinant inbred lines (RIL), F1, F2 and backcross (BC) populations.

Of these mapping populations, RILs offer unique advantages in QTL studies (Burr and Burr 1991, Knapp and Bridges 1990, Austin and Lee 1996). First, a well-characterized RIL population can be permanently propagated and used indefinitely without further genotyping. Second, a trait value for each genotype can be evaluated on several sister plants, which minimizes the environmental variation and improves the accuracy of the QTL analysis. Third, experiments can be replicated over years and environments using identical genotypes thus allowing the detection of QTL that cause genotype-environment interactions. And finally, maps constructed using RILs have a higher genetic resolution as compared to F2 populations (Burr and Burr 1991), as chromosome pairing and recombination during an extended period of inbreeding increases the probability of chromosome recombination. The disadvantage of using RILs is that the development of RILs is time consuming and not readily accessible to self-incompatible species like cultivated potato. Furthermore, the dominant effects at QTL cannot be estimated as done in F2. RILs have been used in maize, rice, Arabidopsis, wheat and oat for various studies such as map construction (Cho *et al*. 1998), QTL mapping (Frova

and Gorla 1993, Austin and Lee 1996, Lister *et al*. 1993, Alonso-Blanco *et al*. 1998) and candidate gene analysis (Faris *et al*. 1999, Kianian *et al*. 1999).

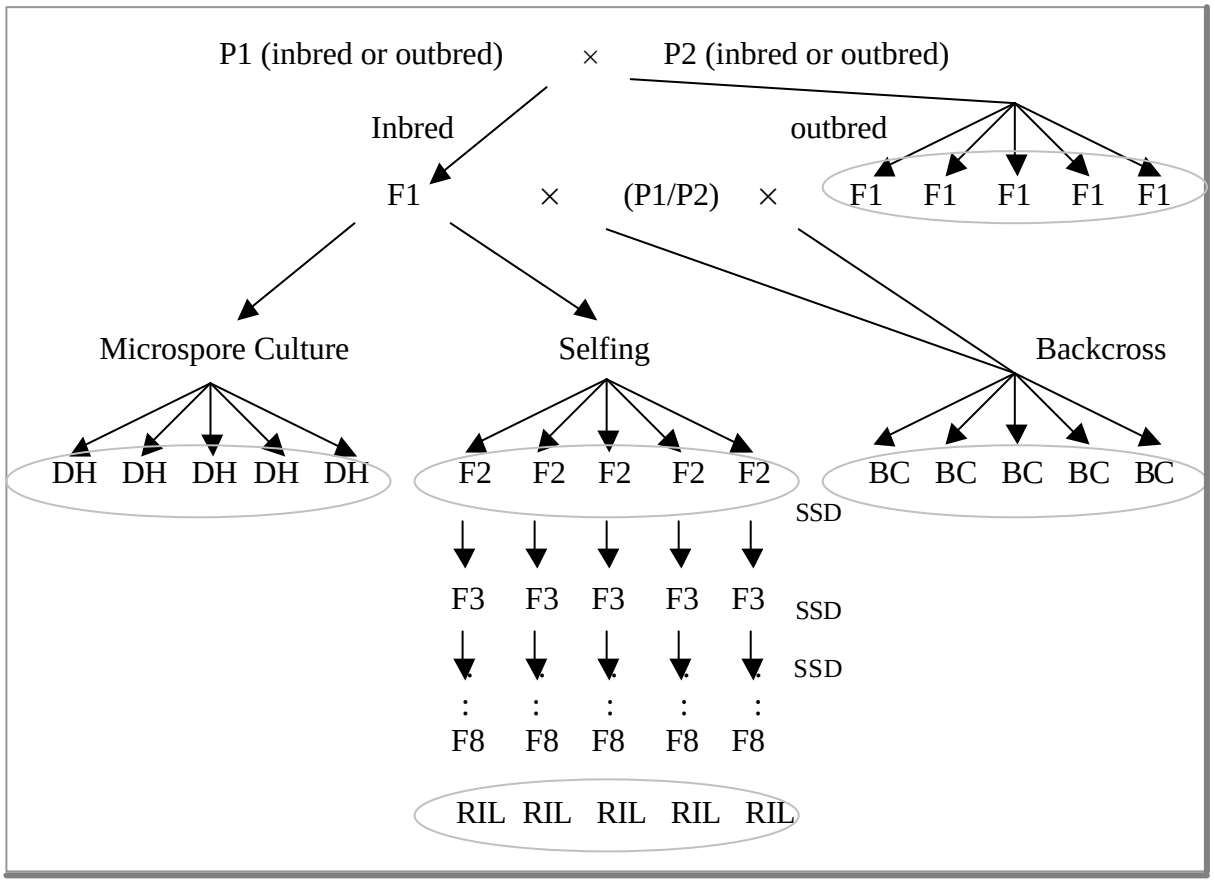


Figure 1-2 Common strategies for the construction of mapping populations. A DH mapping population is generated from pollen of F1 by anther or microspore culture followed by doubling of haploid. Recombinant inbred lines (RIL) are developed by successively selfing single F2 plants by single-seed descent (SSD) method until homozygosity is achieved in the F8 generation. A BC population is derived from the crossing of an F1 individual with one of the parents of F1.

Due to the self-incompatibility of potato at the diploid level and inbreeding depression, the generation of specific genetic stocks like inbred lines for molecular mapping is not applicable. Instead, segregating F1 populations from highly heterozygous parents can be employed for mapping. Such mapping populations, which make efficient use of existing genetic stocks, have been successfully used for linkage analysis in potato (Gebhardt *et al*. 1989, 1991). The estimation of recombination frequencies in such F1 populations has been investigated in detail by Ritter *et al*. (1990).

1.5.2 Molecular marker technologies for genetic mapping

Molecular marker technology has changed dramatically during the past two decades. The first molecular markers were isozyme markers which were based on the different mobility of differently charged protein with the same enzymatic function on the gel. Enzyme markers have limited genome coverage and numbers. Thus, they have subsequently been replaced by DNA-based restriction fragment length polymorphism (RFLP) markers, which reveal neutral sites of variation at the DNA sequence level and are independent of the environment and developmental stages and are not limited in number (Gebhardt *et al*. 1989). After that, the development of PCR-based markers such as RAPD (random amplified polymorphic DNA), AFLP (amplified fragment length polymorphism), and SSR (simple sequence repeats) has greatly accelerated and simplified the mapping process. Currently, the advent of single nucleotide polymorphism (SNP) as a new molecular marker technology has opened a new possibility for plant genome analysis (Cho *et al*. 1999).

RFLP

RFLP was first proposed by Botstein *et al.* (1980) as a new source of genetic markers in humans. This technique involves restriction of genomic DNA followed by electrophoretic size separation of the fragments in a gel matrix. The fragments are then transferred to a membrane by Southern blotting and hybridized with a radioisotope-labelled probe. Many sources of DNA can serve as probes, such as a small piece of genomic DNA, cDNA sequence, or specific PCR products. Sometimes, probes can be adopted from other species as heterologous probes for comparative mapping which allows comparison of genome organization and evolution between the related species (Tanksley *et al*. 1988, Bonierbale *et al*. 1988, Lagercrantz *et al*. 1996, 1998). The polymorphisms detected by RFLP rely on the specific and characteristic nucleotide sequence which is recognised and cut by restriction enzymes. Insertions or deletions between restriction sites or mutations occurring at restriction sites result in length polymorphisms of restriction fragments. The range of fragment length and number of fragments depends on different enzymes. Six-base cutter enzymes generate fragments with 4096 bp in length on average, while a four-base cutter enzymes generates, on average, 256 bp fragments assuming that all four bases are randomly distributed and in equal amounts in the genome. As four-base cutters score more nucleotides per length unit of DNA than six-base cutters (Kreitman and Aquade 1986), therefore, they are more efficient in detecting point mutations. RFLP markers are often co-dominant and, therefore, very informative. However, generating RFLP data is labour intensive and time consuming and requires a relatively large amount of DNA. RFLP maps have been developed for a number of species like maize (Helentjaris *et al*. 1986, Helentjaris 1987), tomato (Bernatzky and Tanksley 1986a, Helentjaris *et al*. 1986, Zamir and Tanksley 1988), lettuce (Landry *et al*.

1987b), rice (McCouch *et al*. 1988), pepper (Tanksley *et al*. 1988), Arabidopsis (Chang *et al*. 1988) and peanut (Halward *et al*. 1992).

In potato, the first RFLP map, which consisted of 135 RFLP markers, was constructed using tomato cDNA and genomic DNA as probes in an interspecific mapping population (Bonierbale *et al*. 1988). This comparative map exhibited high collinearity of marker order in both species. Based on the evaluation of genetic diversity of 38 potato lines of *Solanum tuberosum* by RFLP, Gebhardt *et al*. (1989) demonstrated the feasibility of constructing of a potato RFLP map using an intraspecific mapping population. This RFLP map with 1034 cM in length and 80 % coverage of the genome was developed using a backcross population derived from two of the 38 lines (Gebhardt *et al*. 1991). Further RFLP maps have been constructed using different mapping populations for different purposes in subsequent studies (Gebhardt *et al*. 1991, 1994, Schäfer-Pregl *et al*. 1998).

RAPD

Random Amplified Polymorphic DNA (RAPD) (William *et al*. 1990, Welsh and McClelland 1990) is a PCR-based technique for DNA fingerprinting. The generation of RAPD markers is relying on the probability that 10 bp single primers of arbitrary nucleotide sequence occur on both strands of DNA in inverted orientation within a distance amplifiable by PCR. RAPD markers are technically easy to detect, only small amount of DNA and no prior sequence information is needed. RAPDs can rapidly be used to construct linkage maps. These advantages have allowed mapping of a wide variety of plant genomes (Devos and Gale 1992, Waugh and Powell 1992, Tingey and Del Tufo 1993). RAPD markers were also instrumental for map-based cloning of disease-resistance genes (Jones *et al*. 1994, Mindrinos *et al*. 1994, Whitham *et al*. 1994, Martin *et al*. 1991, Michelmore *et al*. 1991). However RAPD markers are inherited usually in a dominant manner, are not transferable from one population to another and are poorly reproducible between different laboratories (Penner *et al*. 1993, Jones 1997). Consequently, RAPD is largely being replaced by a more robust DNA fingerprinting technique termed amplified fragment length polymorphism (AFLP).

AFLP

AFLP is a relatively new PCR-based DNA fingerprint technique (Vos *et al*. 1995, Zabeau and Vos 1993). It involves restriction of genomic DNA followed by ligation of adaptors to restricted fragments and preselective and selective PCR amplification of a subset of these fragments. The amplified fragments are resolved on a sequencing gel and visualised either by autoradiography or fluorescent sequencing equipment or fluorescent scanner (Meksem *et al*. 1995, Zhang *et al*. 1999, Schwarz *et al*. 1999, Huang *et al*. 2000, Huang and Sun 1999, Hartl and Seefelder 1998), depending on the method of labelling or silver ***(continue to page 9)***

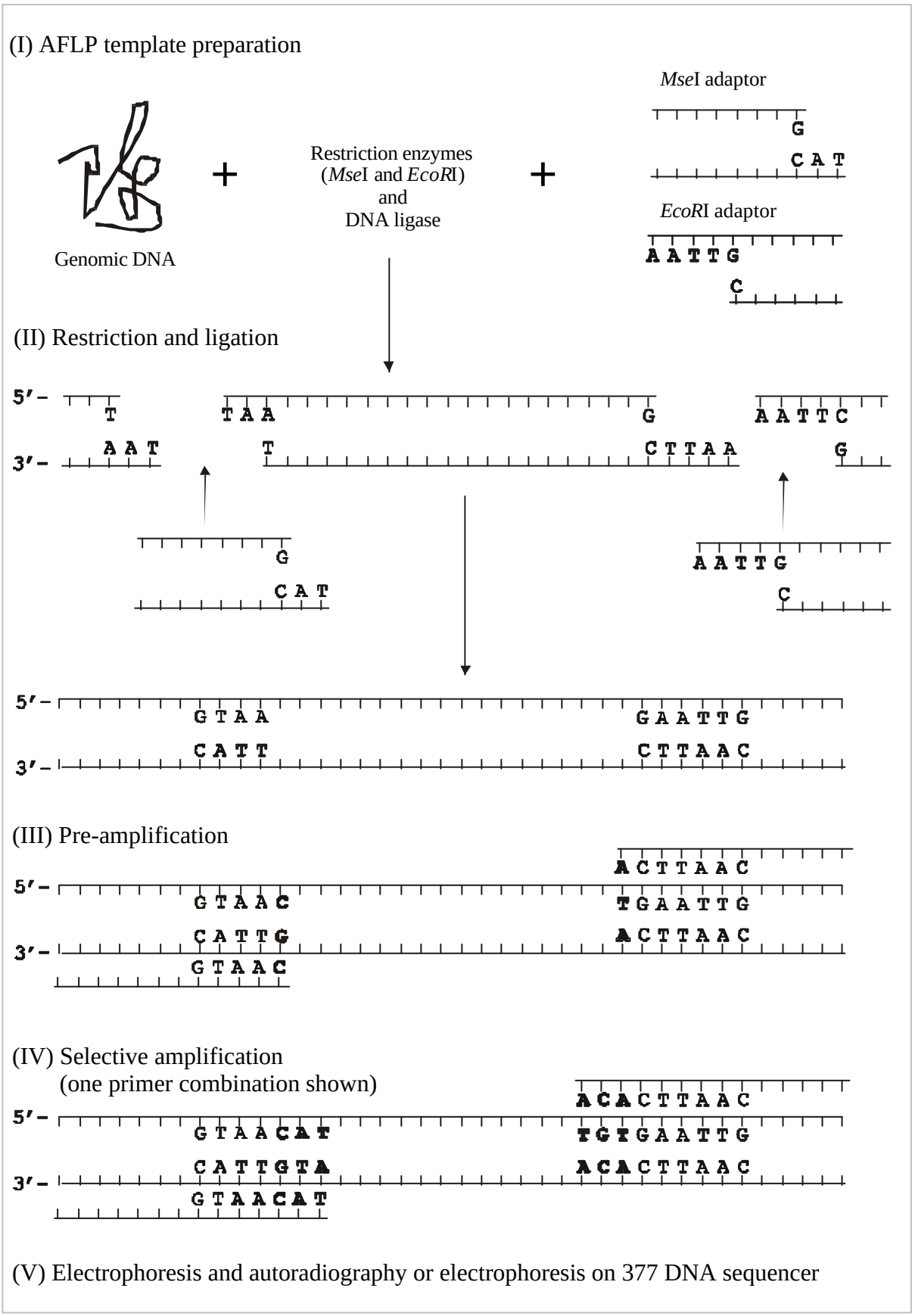


Figure 1-3 Schematic representation of AFLP (modified based on Mueller *et al.*1999)

(continued from page 7)

staining (Cho *et al*. 1996). Most typically, visualisation of AFLPs is achieved by incorporating a $^{33}PO_4^-$ group from [γ^{33}-P]-ATP into a dephosporylated 5' end of one primer. This process, which involves radioactivity, was termed in the present study as radiolabelled AFLP (rAFLP). More recently, an improved AFLP technique, which was developed by Applied Biosystems (ABI), is performed by using fluorescence-labelled primers in combination with an automated sequencing system and software packages for data collection and scoring. The fluorescence-based detection of AFLPs (fAFLPs) offers a great improvement in fragment scoring and data handling. However, the few studies up to date using fAFLP as a tool are limited to genetic diversity study (Zhang *et al*. 1999), saturation mapping of a target region (Schwarz *et al.* 1999) and the chromosome assignment of fAFLP markers (Huang *et al*. 2000). The diagram summary of the AFLP technique is depicted in Figure 1-3 (on page 8).

The notable advantage of AFLP is its capacity to analyse a large number of polymorphic loci simultaneously throughout the genome with a single primer pair on a single gel without prior sequence knowledge. In contrast to RAPD, AFLP is highly reproducible and also transferable between different populations (Jones 1997, Yin *et al*. 1999, Waugh *et al*. 1997, Li *et al*. 1998, Rouppe van der Voort *et al.* 1997). AFLP has been used effectively in a variety of organisms including bacteria (Keim *et al.* 1997), fungi (Rosendahl *et al*. 1997, Van Der Lee *et al*. 1997), animals (Ajmone-Marsan *et al*. 1997, Liu *et al*. 1998) and plants. One major limitation of AFLP is the dominant nature and the difficulty in identifying allelic variants at a specific locus although codominant AFLP markers have been found, however, in frequencies of 4-15 % among all polymorphic AFLP markers (Waugh *et al.* 1997, Lu *et al.* 1998, Boivin *et al.* 1999). Furthermore, AFLP banding pattern may be affected by differential methylation of DNA in different organs from which the template DNA was extracted (Donini *et al*. 1997).

AFLP is now the first option to saturate a particular region of the genome when map-based cloning is applied to cloning target genes. AFLP has also been used in phylogenetic studies based on measures of genetic distance (Pakniyat 1997, Powell *et al*. 1996, Tohme *et al*. 1996, Russell 1997) and for identification of varieties (Schut *et al*. 1997). In addition, it has also been reported to monitor the differential expression of genes using cDNA from different plant organs (Bachem *et al*. 1996, Hartings 1999). Above all, one major application of AFLP is for molecular genetic mapping. It has been used to construct maps for barley (Becker *et al.* 1995, Waugh *et al*. 1997, Qi *et al.* 1998), sugar beet (Schondelmaier *et al*. 1996), soybean (Keim *et al*. 1997), petunia (Gerats *et al*. 1995), rice (Maheswaran *et al*. 1997) and tomato (Haanstra *et al*. 1999).

In potato, using 6 primer combinations of *Eco*RI/*Mse*I primer set and a diploid mapping population, Van Eck *et al*. (1995) constructed an AFLP map which consisted of 264 AFLP

markers and had a map length of 1170 cM. Meyer *et al*. (1998) performed the first experiment of molecular mapping in tetraploid potato using a tetraploid breeding family in combination with AFLP marker technology. The linkage map consisted of 231 maternal and 115 paternal AFLP markers with a total map length of 991 cM and 485 cM, respectively, representing approximately 25 % coverage of the tetraploid genome.

SSR

Simple sequence repeats (SSR), also termed microsatellite or short tandem repeats (STR), are di-.tri-, tetra- or pentanucleotide tandem repeats that are dispersed throughout the genome (Tautz 1989, Weber and May 1989). The basis of polymorphism lies in the variation of the number of tandemly repeated short nucleotide motifs which can be detected by PCR amplification (Saiki *et al*. 1988) using sequences flanking the repeats as primers. Microsatellite markers are inherited in a Mendelian fashion, they are codominant hypervariable (Saghai Maroof *et al*. 1994) and transferable within species from which primers were developed. SSRs have been used for the construction of linkage maps in *Arabidopsis* (Bell and Ecker, 1994), soybean (Akkaya *et al*. 1995), and maize (Senior *et al*. 1996). SSRs have also been used to study the allelic profiles of genotypes for purposes of genotype identification in potato (Kawchuk *et al*. 1996, Schneider and Douches 1997), soybean (Morgante and Olivieri 1993, Cregan *et al*. 1994, Rongwen *et al*. 1995, Maughan *et al*. 1995), grape (Thomas and Scott 1993) and rapeseed (Kresovich *et al*. 1995)

SNP

SNPs, single nucleotide polymorphisms, are single base pair positions in genomic DNA at which different sequence alternatives (alleles) exist in normal individuals in natural populations (Brookes 1999). Significant efforts towards large-scale characterisation of SNPs were first initiated in human genome research. SNPs have since been shown to be the most common type of genetic variation in organisms. Of all the different types of sequence change including single nucleotide substitutions, insertions/deletions and copy number variation in nucleotide repeat motifs, SNP represents about 90 % of human DNA polymorphism (Collins *et al*. 1998). SNPs have been found to occur with a frequency as high as 1 in every 202 bp in the mouse genome and 1 in 1000 bp in the human genome (Lindblad-Toh *et al*. 2000, Wang *et al*. 1998).

There are various methods for SNP detection and scoring. More commonly used are gel-based sequencing and high-density variation-detection DNA chips (VDAs) (Wang *et al*. 1998). In the gel-based sequencing method, sequence trace data are compared using the computer program *Phred* for base calling, *Phrap* for sequence assembly and *Consed* for sequence assembly editing (Nickerson *et al*. 1997). The VDA technique involves

hybridisation to high-density DNA probe arrays or DNA chips (Wang *et al*. 1998). In addition, public EST databases can be used directly to identify SNPs by comparing sequences from different sources (Picoult-Newberg *et al*. 1999). Similarly, various techniques have been invented for genotyping SNP on large scale. The most common ones are 1) microarrays (chips) by hybridisation to allele-specific oligos (Hacia *et al*. 1999), 2) MALDI-TOF (matrix-assisted laser desorption ionisation mass spectrometry with time of flight) by detection of different molecular masses caused by the SNPs (Haff and Smirnov 1997), 3) minisequencing/single nucleotide primer extension by incorporation of specific fluorescence-labelled ddNTPs at the SNP site (Syvanen 1999). Moreover, single strand conformation polymorphism (SSCP) (Orita *et al*. 1989) and heteroduplex (HD) (White *et al*. 1992), both of which are gel-based techniques to detect conformational polymorphisms of DNA, can also be used to detect SNPs with high sensitivity (Ganguly *et al*. 1993, Sheffield *et al*. 1993) and specific mutated nucleotides showing as SNPs can be readily determined by sequencing (Schneider *et al*. 2000). In addition, the allele-specific SCAR marker is actually the gel-based detection of SNPs at sites where primers were designed for detecting specific alleles. This has been used for molecular marker conversion and mapping (Van Heusden *et al*. 1999).

SNP has many advantages and great potential for many applications. Since SNPs exist over the whole genome of organisms with a relatively high frequency, they could facilitate the development of highly dense genetic function maps that would be highly valuable for genome analysis. Moreover, as the sequence context of the SNPs is already known, it has the potential for automation and can facilitate the genetic study of associating sequence variations with heritable phenotypes on a large scale. Because of this, there has recently been considerable interest in SNP discovery and detection for genome analysis of plant (Cho *et al*. 1999)

1.5.3 Applications of molecular genetic map

The invention of molecular marker technology such as RFLP, RAPD, AFLP, and SSR as outlined in the previous sections has opened up a new era for genetic analysis of plant genomes. Genetic mapping using molecular marker technology is of great significance to plant breeding, plant genetic and evolutionary studies. The most common applications of genetic linkage maps are concentrated on the following areas. First, genetic linkage map can be used for marker-assisted selection (MAS) in plant breeding. Linkage maps could help identification of DNA markers linked to single genes of major agronomic importance and the tightly linked DNA markers can be used as diagnostic tools for MAS. This is particularly suitable and powerful for screening for monogenic disease resistance. One of the successful examples is MAS for soybean cyst nematode resistance (SCN) (Cregan *et al*. 1999). The SSR marker Satt309, which is located 1-2 cM away from the gene *rhg*1 for resistance to SCN, has

been developed and used for tagging and tracking the gene through breeding programs, leading to the development of resistant lines. The use of SSR markers has largely decreased the time and effort involved as compared to phenotypic selection. Second, genetic linkage maps can be used for the genetic analysis of quantitative traits (see Sect. 1.7.1.1). With the construction of molecular linkage maps, characterization of quantitative traits has been greatly facilitated in identifying the genomic regions responsible for the traits, postulating the types of gene action and determining the role of epistatic effects and estimating the possible number of genetic factors controlling the traits of interest (Tanksley 1993). Third, genetic linkage mapping can be used to correlate the phenotypic traits with the genes controlling the trait, which includes map-based cloning of a gene of known heritable phenotype and postulating candidate genes for a trait with known biochemical basis. Finally, genetic linkage maps provide insights into chromosomal organization and could be useful in map-based evolutionary studies by comparative mapping.

With respect to potato, a variety of studies have been accomplished using potato molecular mapping as a tool. Many genes controlling monogenic traits have been positioned on to the potato linkage map. The majority are single genes for resistance to various pathogens. *Rx1*, *Rx2*, *Nb* and *Rx*, which condition resistance to potato virus X (PVX), were mapped on linkage groups XII and V (Ritter *et al.*1991, De Jong *et al*. 1997, Bendahmane *et al*. 1997). *Ry* for resistance to potato virus Y (PVY) were on linkage group XI (Brigneti *et al*. 1997, Hamalainen *et al*. 1997). Five out of 11 known single dominant factors conferring race-specific resistance to *P. infestans* have been localised, *R1* on linkage group V (Leonards-Schippers *et al*. 1992, El-Kharbotly *et al.* 1994), *R3*, *R6* and *R7* on linkage group XI (El-Kharbotly *et al*. 1994, 1996a) and *R2* on linkage group IV (Li *et al* 1998). *Gro*1, *H*1 and *Gro*VI conferring resistance to *Globodera rostochiensis* were mapped on to linkage group VII and V (Barone *et al*. 1990, Gebhardt *et al*. 1993, Jacobs *et al*. 1996). The *Gpa*2 gene for resistance to *Globodera pallida* was mapped on to linkage group XII (Rouppe van der Voort *et al*. 1997). Some tuber morphological traits were also mapped including tuber flesh colour *Y* on linkage group III (Bonierbale *et al*. 1988), flower colour loci *D*, *F*, and *P* on linkage group II, X and XI, respectively (Van Eck *et al*. 1993), tuber shape *Ro* on linkage group X (Van Eck *et al*. 1994a), tuber skin pigmentation *PSC* on linkage group X (Van Eck *et al.* 1994b, Gebhardt *et al*. 1991), tuber storage protein *Patatin* on linkage group VIII (Ganal *et al.* 1991, Gebhardt *et al*. 1991). In addition, a number of cloned genes of known function have also been mapped (Gebhardt *et al*. 1994).

1.6 Function mapping

Anonymous molecular markers such as RFLP, SSR, RAPD, AFLP usually have no known function. However, candidate genes analysis for QTL uses known genes as markers and

identifies their map position on chromosomes. To this end, several approaches can be employed. Southern hybridization with a cloned gene as probe is one approach to localize the gene on molecular linkage map (Gebhardt *et al*.1994). Alternatively, linkage maps based on expressed sequence tags (ESTs) are considered as done in rice (Kurata *et al.* 1994) and maize (Chao *et al*. 1994). This depends, however, on the availability of cloned genes and a hybridization-based marker is not suitable for marker-assisted selection (MAS) screening of plants on a large-scale.

In the past decade, intensive research in plant molecular biology has led to the constant and daily increase in the number of gene sequences becoming available in databases. For example, in potato, more than 4300 sequence entries (until writing the thesis) are present in the GenBank-EMBL sequence databases, of which at least 70 genes are directly or indirectly involved in carbohydrate metabolism and transport. These metabolic pathways are physiologically and biochemically related to agronomic traits of potato such as starch and reducing sugar content of tubers. It is thus timely to explore the possibility of using sequence sources from public databases for mapping functional genes that are of agronomic interest and, in parallel, to develop STS-based and gene-specific markers.

Although many genes have been cloned and sequenced from plants and their number increases, the number of known genes available for a given species or a given pathway is still limited. This can be compensated by the use of amplified consensus genetic markers (ACGM) (Brunel *et al*. 1999). This approach takes advantage of high conservation of genes between related species and uses consensus sequence motifs as primers to amplify analogous sequences (Schneider *et al*.1999, Van Campenhout 2000). Another approach for complementing a set of functional genes is the use of ESTs. The putative function of expressed sequence tags (ESTs) can be deduced from sequence similarity to known genes from bacteria, animals or plants by database searching. Much effort has been spent on ESTsequencing of cDNA in *Arabidopsis*, rice and tomato (Sasaki *et al*. 1994, Delseny *et al*. 1997, Ganal *et al*. 1998).

1.6.1 Metabolic pathways relevant to starch and sugar content of potato tubers

The amount of starch accumulated in mature potato tubers is the net result of a chain of biochemical processes starting from photosynthetic carbon fixation followed by synthesis of transient starch and conversion into sucrose in photosynthetically active source leaves, vascular transport of sucrose from the leaves to the developing sink tuber and starch synthesis and degradation in the tuber during the growth period. These processes are biochemically related to carbohydrate metabolism and transport which include, among others, the Calvin

cycle, the oxidative pentose phosphate pathway, glycolysis, the TCA (tricarboxylic acid) cycle, sucrose metabolism and transport, and starch metabolism.

1.6.1.1 The Calvin cycle

The Calvin cycle also known as the C3 cycle or reductive pentose phosphate pathway (Calvin and Bassham 1962), is the second phase of the dark reactions of photosynthesis. During photosynthesis, the biological energy forms (ATP and NADPH) which were converted from solar radiation in the photosynthetic light reaction, are used for fixation of carbon dioxide. The Calvin cycle has three phases including carboxylation, reduction and regeneration of sugar intermediates. The products are triose phosphates and 3-phosphoglycerate which are either transiently converted into starch by a series of reactions in chloroplast or exported to the cytosol for further metabolism such as respiration and sucrose biosynthesis. The enzymes and reactions involved are listed in Table 1-3 (on page 15) and schematically shown in Figure 1-4 (on page 16).

1.6.1.2 Respiratory pathways

Glycolysis

Glycolysis provides the cell with ATP and the reducing power of NADH, the cofactors to satisfy the biosynthetic needs particularly in nongreen tissues such as the fatty acid biosynthesis in leucoplasts (Dennis *et al*. 1997) Glycolysis also provides intermediates, by oxidation of glucose to pyruvate, for other biosynthetic pathways such as the synthesis of polysaccharides and lignin which are the components of cell walls (Dennis *et al*. 1997). Two glycolytic pathways exist in plants, one in the cytosol and one in plastids. These two pathways communicate through the transporters of the inner plastid envelope (Kammerer *et al*. 1998, Dennis *et al*. 1997). The enzymes catalysing the reactions within plastids are isozymes of their cytosolic counterparts (Dennis *et al*. 1997) (see Table 1-3, Figure 1-4, on page 15-16).

Oxidative pentose phosphate pathway

The oxidative pentose phosphate pathway occurs, as glycolysis, in both the cytosol and in plastids. The function of this pathway is to generate the cofactor NADPH and intermediates for other biosynthetic pathways such as the biosynthesis of nucleic acids, amino acids, the polyphenols and lignin (Dennis *et al*. 1997). The oxidative pentose phosphate pathway consists of two parts, the oxidative irreversible section, which is catalysed by G6PDH and PGDH, producing NADPH, and a reversible section that is involved in the regeneration of

hexose from ribulose 5-phosphate by the action of ribulose 5-phosphate epimerase, ribose 5-phosphate isomerase, transketolase, and transaldolase. The enzymes catalysing the reactions in the cytosol are isozymes of their plastidic counterparts (see Table 1-3 and Figure 1-4).

Table 1-3 Major known enzymes involved in carbohydrate metabolism and transport (see Figure 1-4 for functions)

	Calvin Cycle
1	ribulose bisphosphate carboxylase
2	phosphoglycerate kinase
3	glyceraldehyde-3-phosphate dehydrogenase
4	triosephosphate isomerase
5	plastidic aldolase
6	chloroplast fructose1,6-bisphosphatase
7	transketolase
8	transaldolase
9	sedoheptulose-bisphosphatase
10	pentose-5-phosphate 3- epimerase
11	ribose-phosphate isomerase
12	phosphoribulokinase
	Glycolysis
13	hexokinase
14	phosphoglucomutase
15	phosphoglucose isomerase
16	phosphofructokinase
17	aldolase
18	triosephosphate isomerase
19	glyceraldehyde 3-phosphate dehydrogenase
20	phosphoglycerate kinase
21	phosphoglycerate mutase
22	enolase
23	pyruvate kinase
	TCA Cycle
24	pyruvate dehydrogenase
25	citrate synthase
26	aconitase
27	isocitrate dehydrogenase
28	α-ketoglutarate dehydrogenase
29	succinate thiokinase
30	succinate dehydrogenase
31	fumarase
32	malate dehydrogenase
33	NAD-malic enzyme
	Pentose Phosphate Pathway
34	glucose-6-phosphate dehydrogenase
35	gluconate-6-phosphate lactonase
36	6-phosphogluconate dehydrogenase
37	phosphopentoisomerase
38	phosphopentoepimerase
39	transketolase
40	transaldolase
	Sucrose Metabolism
41	pyrophosphate-fructose-6-phosphate-1-phosphotransferase
42	fructose 1,6- bisphosphatase
43	UDP-glucose pyrophosphorylase
44	sucrose -phosphate synthase
45	sucrose phosphatase
46	sucrose synthase
47	soluble acid invertase
48	fructokinase
49	sucrose transporter
50	H^+-ATPase
51	apoplastic invertase
52	glucose transporter
53	glucose-6-P transporter
	Starch Metabolism
54	ADP-glucose pyrophosphorylase
55	soluble starch synthase
56	granule bound starch synthase
57	4-α -glucanotansferase (D-enzyme)
58	branching enzyme
59	L-type starch phosphorylase
60	α-glucan phosphorylase
61	debranching enzyme
62	α-amylase
63	β-amylase
64	α-glucosidase
65	hexokinase
66	phosphoglucomutase

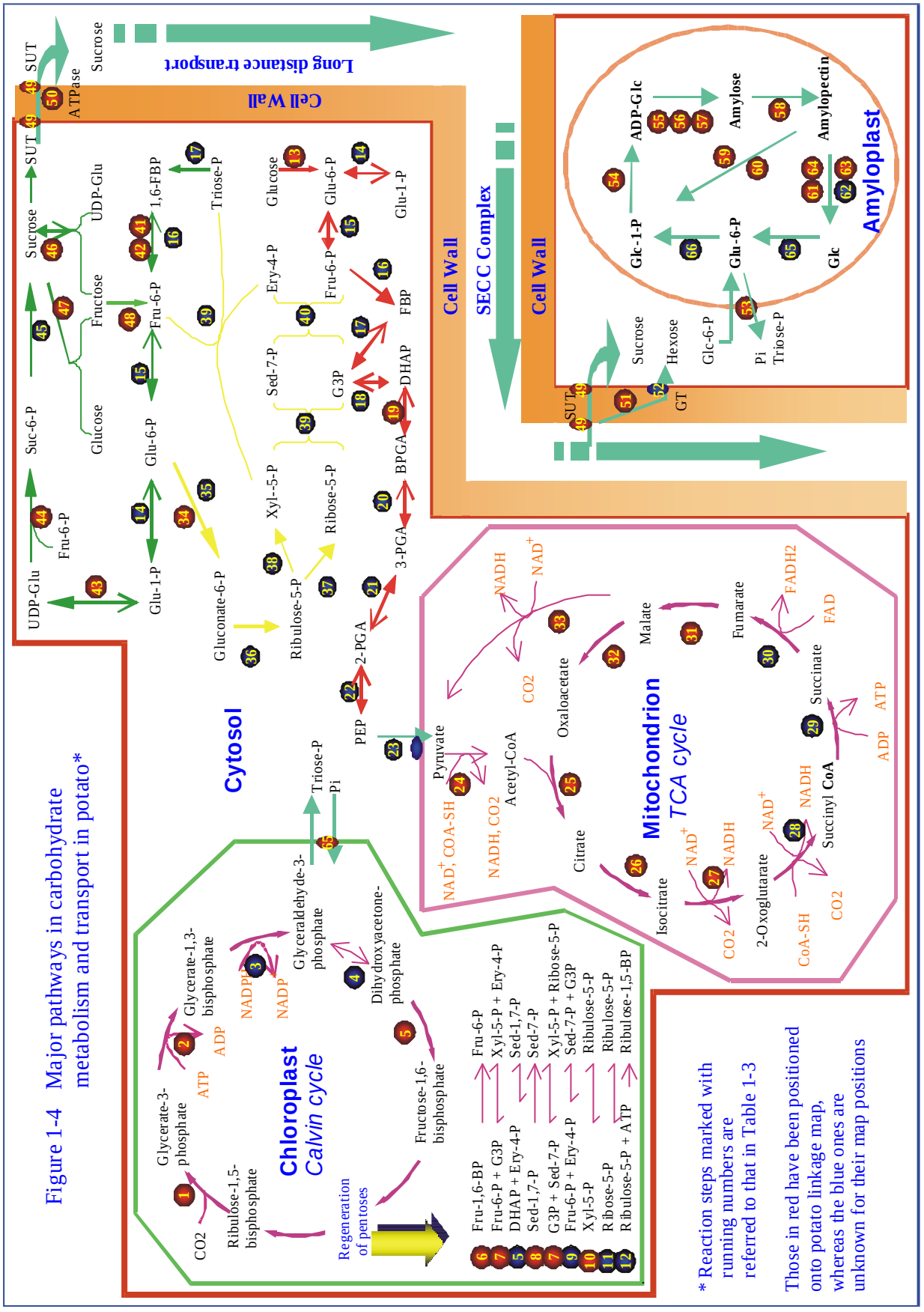


Figure 1-4 Major pathways in carbohydrate metabolism and transport in potato*

References: Frommer and Sonnewald (1995), Quick *et al*. (1997), Dennis *et al*. (1997), Zubay (1998)

(continued from page 14)

The oxidative pentose phosphate pathway is intimately linked to glycolysis. These two pathways share three common intermediates: glyceraldehydes 3-phosphate, fructose 6-phosphate and glucose 6-phosphate. The metabolic flow through either of the pathways is determined by the metabolic needs of the cell. In general, carbon flow through the oxidative pentose phosphate pathway is relatively small as compared to other respiratory pathways such as glycolysis and TCA cycle (Dennis *et al*. 1997).

TCA (tricarboxylic acid) cycle

The TCA cycle, alternatively known as the citric acid cycle or Krebs cycle, occurs in mitochondria with pyruvate and malate as the main substrates. It operates as a catabolic pathway oxidizing a major portion of carbohydrates, lipids and amino acids. It has an important role in the biosynthesis of organic acids and amino acids which are the precursors for many biosynthetic pathways. In the anabolic process, the intermediates are replenished through the synthesis of oxaloacetate from phosphoenolpruvate by phosphoenolpruvate carboxylase (Hill 1997). Usually, important developmental stages of the plant life cycle are accompanied by increases in the rate of respiration and are often associated with increased biosynthesis of cellular constituents (Hill 1997).

1.6.1.3 Sucrose metabolism

Sucrose is the principal product of photosynthesis and the major form for transport and storage in plant. Sucrose synthesis operates in the cytosol via the pathway involving the enzymes: UGPase, sucrose phosphate synthase and sucrose phosphatase (Kruger 1997). Sucrose breakdown is catalysed by sucrose synthase and invertase. The former one catalyses the interconversion between sucrose, UTP, UDP-glucose and fructose, and the latter one catalyses the irreversible hydrolysis of sucrose to glucose and fructose (Table 1-3, Figure 1-4, on page 15-16).

1.6.1.4 Starch metabolism

Potato starch is a mixture of amylose and amylopectin which exist in semi-crystalline granules in plastids (amyloplasts). Amylose contains 600-3000 1,4-α-glucosyl residues with a 1,6-α-glucosyl branch every 1000 residues. Amylopectin contains 6000-60000 glucosyl residues with an average of one 1,6-α-glucosyl linkage every 20 to 26 units (Kruger 1997). Starch synthesis involves AGPase, starch synthases and starch branching enzymes, which are all present in multiple forms in the plant. AGPase is a tetrameric enzyme comprising of two distinct polypeptides (Muller *et al* 1990, Nakata *et al*. 1991), a large subunit and a small subunit, both of which are encoded by several genes that are differentially expressed in

different tissues (Kruger 1997). Storage tissues contain one or more soluble starch synthases (Abel *et al*. 1996) as well as granule-bound starch synthase (Edwards *et al*. 1995). Similarly, two distinguished classes of starch branching enzyme I and II were identified (Khoshnoodi *et al*. 1996, Larsson *et al*.1998). The activity of branching enzymes, together with that of debranching enzymes, determines the relative proportions of amylose and amylopectin (Kruger 1997).

Starch can be degraded either hydrolytically or phosphorolytically. The former pathway involves enzymes like α-amylase, which catalyses the cleavage of the 1,4-α-glucosyl bond resulting in the production of a mixture of glucose, maltose, maltotriose and a range of branched dextrins. β-amylase can also degrade starch by catalysing the removal of successive maltose units from the non-reducing end of α-glucan chains. The maltose and other short maltosaccharides may be further hydrolysed to glucose by α-glucosidase (maltase) and subsequently phosphorylated by hexokinase. The phosphorolytic pathway involves starch phosphorylase which produces glucose 1-phosphate by successive removal of glucosyl residues at the end of an α-glucan. This enzyme can only metabolise oligosaccharides larger than maltotetraose. Further phosphorolytic cleavage may attribute to the action of a glucosyltransferase such as the D-enzyme which makes more of the glucan accessible to phosphorylase by increasing the degree of polymerization of short oligosaccharides (Kruger 1997). The branch points of starch are removed by a debranching enzyme which catalyses the cleavage of 1,6-α-glucosyl bonds hydrolytically and produces oligosaccharides.

1.6.1.5 Major transporters for carbon exchange between the cytosol and the plastid

Metabolite exchange between the cytosol and plastids has been identified to be achieved by three main groups of plastidic phosphate translocators including triose phosphate/phosphate translocator (TPT) (Flügge and Heldt 1991), the phosphenolpyruvate/phosphate translocator (PPT) and the glucose 6-phosphate/phosphate antiporter (GPT) (Kammerer *et al*. 1998).

The principal route for the exchange of carbon between the cytosol and the plastid is the phosphate translocator which exchanges triose phosphate for inorganic phosphate. Other related translocators can transport other metabolites such as hexose phosphates and phosphoenolpyruvate (Borchert *et al*., 1993, Trimming and Emes, 1993). The TPT functions predominantly in photosynthetic tissues, GPT in root and reproductive organs, whereas, PPT appears to be ubiquitously expressed (Kammerer *et al*. 1998).

Sucrose is the major form of photoassimilates for long distance transport from photosynthetic tissues to sink organs. This process involves generally three steps. First, sucrose is exported from the mesophyll cell to the vicinity of the sieve elements in the smallest veins of the leaf (short-distance transport) mainly via the symplast or through the apoplast (Frommer and

Sonnewald, 1995). This process is thought to occur by a passive mass flow. Second, sugars are transported into the sieve elements and companion cells for sieve element loading. The movement of sugars is driven by an active transport by the sucrose transport protein (SUT), from the apoplast against a concentration gradient, into the conducting complex. This is coupled with proton transport. The symport of sucrose and protons is driven by a membrane H^+-ATPase which maintains an electrochemical proton gradient across the membrane. Finally, once inside the sieve elements, sucrose and other solutes are translocated through long-distance transport from the source to sink organs by a pressure flow mechanism.

1.7 QTL analysis

Many traits of agricultural and economical significance exhibit quantitative inheritance, such as yield, substrate content, plant maturity, disease resistance and stress tolerance. Quantitative traits show continuous phenotypic variation in a population resulting from the combined allelic effects of many genes and environmental conditions. The genetic loci which control quantitative traits are referred to as QTL (quantitative trait loci). QTL analysis has been a major area of genetical study for many decades. The earliest documented experiments on linkage analysis between quantitative effects and marker genotypes have been reported by Sax (1923) and Thoday (1961). However, for most of the period up to 1980, the study of quantitative traits has largely involved biometrical approaches based on means, variances, covariances of relatives and, consequently, very little is known about the biological nature of quantitative or natural variation in terms of number and location of the genes that underlie them (Fisher 1918, Wright 1934, Mather 1949, Falconer 1960). It is only during the past decade when the advent of efficient molecular marker technologies as outlined in Section 1.5.2 and specific statistical methods that it was possible to follow the segregation of quantitative traits via linked markers (Tanksley 1993) and to detect effects, numbers and map positions of QTL.

1.7.1 Approaches to QTL analysis

The identification of QTL for economically important traits has been achieved primarily by two approaches, either through linkage mapping to anonymous markers as outlined in Sect.1.5.2. or through association studies involving candidate genes.

1.7.1.1 QTL analysis through a molecular marker approach

Most QTL analyses to date adopt the linkage mapping approach. RFLPs were initially used as markers (Beckman and Soller 1983, Patterson *et al.*1988, Lander and Bostein 1989) followed by PCR-based markers such as RAPD, SSR, and AFLP. The molecular markers,

which cover the total genome, provided the framework map based on which the QTL could be located. The principle of QTL mapping is to associate the phenotypically evaluated trait(s) with molecular markers using statistic tools. The map locations of QTL can then be estimated by the means of highly associated markers. Typically, the detection and location of the loci underlying quantitative trait variation involves three essential steps. First, a segregation population is created and characterized with molecular markers. This usually leads to the construction of a genome-wide genetic map of the population. Second, the individuals of the same population are phenotypically evaluated for the traits under investigation. Finally, genotypic molecular marker are analysed for association with the phenotypic trait data using appropriate statistical methods. This type of QTL analysis can lead to the elucidation of QTL parameters in terms of number, position, effects and interactions between them.

1.7.1.2 QTL analysis through a candidate gene approach

The candidate gene approach correlates a phenotype with its underlying biochemical or physiological basis by demonstrating that candidate genes are tightly linked to the genetic locus of interest. The candidate-gene approach is a powerful and robust method. Compared to the genome wide mapping strategy, the chances of finding markers linked to putative QTL are maximized, since the selection of candidate gene markers is based on known relationships between biochemistry, physiology and the agronomic character under study. This approach has been applied successfully in various QTL analyses, such as mapping QTL for defense response to diseases in wheat (Faris *et al.* 1999, Pflieger *et al.* 1999), for resistance to corn earworm in maize (Byrne *et al.* 1996, 1998) and early growth traits in maize (Causse *et al.* 1995) and bud set and bud flush in *Populus* (Frewen *et al.* 2000). The candidate gene approach is sometimes limited by the understanding of the physiology and biochemistry of the trait of interest and by the requirement of prior identification of genes that potentially involved in the trait expression. The confirmation of the causal relationship between specific QTL and candidate genes has proven to be difficult. Molecular characterization of the QTL of interest is needed to determine whether the trait variation is caused by the alleles at the candidate gene locus or at tightly linked loci and their effects. The most direct confirmation could be accomplished by creating a series of transgenic plants with alterative QTL alleles in a common genetic background and observing the phenotypic effect of each allele. Unlike the genes controlling monogenic traits, the effect of QTL alleles are likely to be more subtle and cannot be phenotypically observable on all transgenic plants. Therefore, a large number of independent transformations is needed to provide a sufficient sample size for statistical test of the difference between transformed and control plants. A more practicable alternative is the detailed analysis in marker-generated near-isogenic lines (NILs). This approach has been used for the identification of an invertase gene (*Inv*) as a QTL controlling sugar content in tomato (Bernacchi *et al.* 1998, Fridman *et al.* 2000).

1.7.2 QTL analysis in potato

The development of molecular linkage maps facilitates the dissection of complex traits into discrete quantitative trait loci or QTL. Thus, quantitative traits could be investigated with respect to number, effect and location of the underlying genetic factors. In potato, a number of agronomically important quantitative traits have been studied by this method such as potato tuberization, tuber shape, tuber dormancy (Van den Berg *et al*. 1996a, b, Simko *et al*. 1997) and specific gravity (Freyre and Douches 1994). One of the most important aspects in potato genetics and breeding is on quantitative disease resistance particularly to late blight. QTL for resistance to late blight (*P. infestans*) have been detected on eleven out of twelve of the potato linkage groups (Bonierbale *et al*. 1994, Leonards-Schippers *et al*. 1994, Oberhagemann *et al*. 1999). Other quantitative traits that are of much significance in potato breeding are tuber starch content, tuber yield, chip colour, and plant maturity. These traits have also been addressed in several QTL mapping experiments. Using two diploid populations, K31 and LH, and RFLP markers, Schäfer-Pregl *et al*. (1998) identified 17 QTL for tuber starch content which were distributed on all of the 12 linkage groups of potato. Also, using diploid segregating potato, Collins *et al*. (1999) and Oberhagemann *et al*. (1999) identified QTL for foliage earliness with a major QTL on linkage group V. Six QTL for chip colour, which reflected the reducing sugar content of tubers, were detected on linkage group II, IV, V, and X (Douches and Freyre 1994).

However, all these experiments outlined above were carried out at the diploid level to avoid the complexity of tetrasomic inheritance of tetraploid potato. The only mapping experiment performed at the tetraploid level was done by Meyer *et al.* (1998) and Bradshaw *et al*. (1998) in analysing QTL for resistance to late blight and *Globodera pallida*. No other published data has been reported using a tetraploid mapping population to identify QTL for agronomically important traits.

1.8 Agronomic traits under investigation

As outlined above, many agronomic traits of potato both qualitative and quantitative have been extensively studied regarding their nature of inheritance and map positions using diploid potato as experimental materials. In the present study particular efforts have been spent, in collaboration with potato breeding company, on the analysis of three traits: tuber starch content (TSC), reducing sugar content (RSC) and foliage earliness (FE).

Tuber starch content (TSC)

Potato tubers are the agronomic product of potato cultivation while starch is the major component of the potato tubers (Sect. 1.1). High starch yield is an important target, therefore,

in potato breeding. This could be achieved by improvement of both tuber starch content and tuber yield. The present study is more focused on the former component.

Reducing sugar content (RSC)

Reducing sugar content is of critical importance to processing quality of potato. High reducing sugar levels result in dark colored chips with an undesirable taste. This is due to a non-enzymic reaction known as the Maillard reaction by which the carbonyl group of the monosaccharide reducing sugars (glucose and fructose) react with the amino groups of free amino acids during the exposure to heat in processing (Habib and Brown 1959, Shallenberger *et al*. 1959). Thus, low reducing sugar content in mature tubers is required for potato cultivars used by the processing industry.

Foliage earliness (FE)

Early maturity is a prerequisite in Middle and Northern Europe where the growing period is limited. This character is determined by different phenotypic aspects such as earliness of flowering time, tuber initiation and maturity. In potato breeding, earliness is, in general, visually evaluated as foliage earliness based on the vegetative growth of a plant.

1.9 Outline of the thesis

The aim of this thesis was, first, to develop a molecular function map for potato using the diploid potato as a model system with emphasis on functional genes involved in carbohydrate metabolism and transport. The molecular function map was to be used for the identification of candidate genes controlling tuber starch content for which QTL have been mapped previously (Shäfer-Pregl *et al*. 1998). Second, to explore the possibility of discovering single nucleotide polymorphisms (SNPs) in candidate genes by sequencing directly PCR products. Third, to tag QTL for tuber starch content, reducing sugar content and foliage earliness in a tetraploid breeding family through the fAFLP approach and to clone the most significant markers via rAFLP. And fourth, to analyse the QTL through a candidate gene approach based on the function map. To achieve these goals, various experiments were performed:

Nucleotide sequence databases EMBL and GenBank were mined for sequence information of genes of potato and other plant species. Exploration was focused on genes involved in carbohydrate metabolism and transport. A sequence-tagged site (STS) approach was adopted to amplify by PCR homologous or analogous genes in potato. Polymorphisms of the amplification products were revealed either directly as SCAR (sequence characterized amplification region) or as CAPS (cleaved amplified polymorphic sequence) after cleavage with restriction enzymes in two diploid mapping populations K31 and LH. A function map containing 56 functional genes was constructed in conjunction with a previously generated

RFLP marker framework. The comparison of map positions of the functional genes with position of previously mapped QTL using the same populations lead to the identification of putative candidate genes responsible for the phenotypic variation of tuber starch content

SNPs in the candidate gene *Stp*23 were exploited by sequencing PCR products from 14 different potato lines.

QTL tagging by fAFLP was performed using a fluorescent sequencing system. Cloning of significant fAFLP fragments was carried out via rAFLP.

Five genes, which are overlapped with QTL for starch content on linkage map of K31, were selected to test their effects on traits investigated in population Z3.

2. MATERIALS AND METHODS

2.1 Chemicals, enzymes and oligonucleotides

The restriction enzymes were obtained from Boehringer-Mannheim (10 U/µl) currently renamed as Roche (Mannheim), Biolab (England), the *Taq*-Polymerase were purchased from Life Technologies (Freiburg) or Sigma (Deisenhofen) and used with the 10 × buffer supplied, unless otherwise stated.

Laboratory reagents were obtained from following companies: Life Technologies, Pharmacia (Freiburg), Sigma (Deisenhofen), Merck (Darmstadt), Difco (USA), Roth (Karlsruhe), Serva (Heidelberg), unless otherwise stated. Filter membranes were obtained from Whatman. All types of kit were from Qiagen (Hilden) including gel-extraction kit (Qiaex II, Qiaquick). Plant genomic DNA extraction kit (DNeasy Plant Mini) and plasmid Mini and Midi Kit (QiaPrep). dNTP were from Pharmacia. Radioisotopes [α^{32}P]-dCTP (10 µCi/µl) and [γ^{33}P]-ATP (10 µCi/µl) were purchased from Amersham Buchler (Braunschweig).

Oligonucleotides were custom synthesized by MWG-Biotech (Munich), Life Technologies (Freiburg) and ABI-PE (Weiterstadt).

2.2 Plant material

Diploid Mapping populations: K31 and LH

K31 consisting of 157 lines is an outbred diploid F1 population developed at the Max-Planck-Institute in Cologne from a cross between diploid lines: P3 (H80.557/1, *Solanum tuberosum* ssp. *tuberosum*.) as female parent and P38 (H80.576/16, *Solanum tuberosum* ssp. *tuberosum*.) as male parent (Gebhardt *et al*. 1989).

LH was derived from crossing a diploid interspecific hybrid between T710 (S. *chacoense*) as female parent and 45c3 (*S. tuberosum*) as male parent. This population consists of 49 individuals with 24 being high-starch genotypes (tuber starch content 23 % - 33 % on the basis of fresh weight) and 25 low-starch genotypes (11 % - 15 %) which have been selected from 451 F1 hybrids. For both of the populations K31 and LH, a RFLP map has been constructed and QTL analysis for tuber starch content and tuber yield has been performed (Schäfer-Pregl *et al*. 1998).

Tetraploid hybrid family: Z3

Z3 was a tetraploid F1 population consisting of 200 individuals which was derived from the cross between Elles (S3) and 90-313-1 (S1). Population Z3 was provided and developed by SaKa-Ragis Pflanzenzucht GbR (Dr. Josef Strahwald, Windeby).

Potato lines for detecting single nucleotide polymorphisms (SNPs)

Diploid lines: P3, P38, 45C3, T710.
Tetraploid lines: S1, S2, S3, Impala-L, Impala-S, Prior, Bodenkraft, Hansa, Quarta and Taiga.

2.3 Bacterial strain and cloning vector

The genotype *of Escherichia coli* strain TG2: *supE hsdD5 thi D(lac-proAB) D(srl-recA)306::Tn10(tet^r) F'*[*traD36 proAB$^+$ lacI^q lacZDM15*] (Life Technologies).

The *E. coli* plasmid vector pGEM®-T that contains *pMB1*-ori, *amp^r* and is present in 300-400 copies per cell was used for cloning. This system provides blue and white selection for the transformant because the insertion of the cloned DNA interrupts the *lac*Z gene. The vector also contains the T7 and SP6 promoters that can serve as a priming site for insert amplification.

2.4 Media

Luria-Bertani (LB)

10 g/l tryptone 5 g/l yeast extract 5-10 g/l NaCl (15 g/l agar) for LB plating

Add distilled water and adjust pH to 7.0 with NaOH, bring to 1 liter with deionised water. Autoclave at 120ºC for 20 min. For plating, 15 g agar was added before autoclaving. After autoclaving, cool to 50ºC, and then pour the plates either directly without adding ampicillin, or alternatively, with ampicillin added to a final concentration of 100 µg/ml.

SOC medium (100 ml)

2.0 g tryptone 0.5 g yeast extract 1 ml 1 M NaCl 0.25 ml 1 M KCl 1 ml 2 M Mg^{2+} stock solution 1 ml 2 M glucose

Add tryptone yeast extract, NaCl and KCl to 97 ml distilled water, stir to dissolve. Autoclave and cool to room temperature. Add filter sterilised Mg^{2+} stock solution and filter sterilised glucose, each to a final concentration of 20 mM. Bring to 100 ml with sterile distilled water.

2 M Mg^{2+} stock solution (100 ml)

20.33 g $MgCl_2 \bullet 6H_2O$
24.65 g $MgSO_4 \bullet 7H_2O$

Add distilled water to 100ml, filter sterilise.

White and blue selection medium (1 liter)

1000 ml LB with 15 g agar
2 ml 50 mg/ml Ampicillin
2 ml 20 mg/ml X-gal
40 µl 200 mg/ml IPTG

Make the LB plating medium as above, supplement with ampicillin, X-gal and IPTG when the medium is cooled to 50°C, and then pour the plates.

2.5 Buffers and solutions

50 × TAE

2 M Tris-HCl
50 mM EDTA
57.1 ml glacial acetic acid

Adding distilled water to 1 liter, adjust pH to 8.0

$TE_{10/0.1}$

10 mM Tri-HCl
0.1 mM EDTA solution pH 8.0

10 × TBE for RFLP

890 mM Tris-HCl
890 mM boric acid
25 mM EDTA solution pH 8.0

10 × TBE for rAFLP

1 M Tris-HCl
1 M boric acid
20 mM EDTA
solution pH 8.3 (without adjusting)

0.5 × TBE buffer was used as running buffer for rAFLP polyacrylamide-gel electrophoresis.

30% Acrylamide

29.2 % (w/v) acrylamide,
0.8 % (w/v) N-N'-methylene bisacrylamide
dissolved in deionised water

rAFLP gel solution

5 % acrylamide/bisacrylamide (20:1)
7.5 M urea
0.5 × TBE

20 × TPE buffer (5 liters)

160 mM Tris (96.5 g)
4 mM EDTA (7.5 g)
pH 8.3 (*with phosphoric acid*)

0.4 × TPE was used for electro-blotting

20 × SSC

3 M NaCl
300 mM sodium citrate

20 × SSPE

200 mM disodium hydrogen phosphate
20 mM sodium dihydrogen phosphate
3.6 M NaCl
20 mM EDTA pH 8.0

2.6 DNA extraction

2.6.1 Plant genomic DNA extraction

Plant growing and leave material preparation

For each of the Z3 individuals, a single tuber was planted in a 2-liter pot in the greenhouse under normal daylight conditions. 10 g potato leaves are harvested, frozen in liquid nitrogen, freeze-dried and then stored at –20 °C for later use.

Genomic DNA extraction

10 mg freeze-dried leaf material was grinded under liquid nitrogen to fine powder. Total genomic DNA was extracted using DNeasy™ Plant Mini Kit (Qiagen) following the supplier's instructions. Extracted DNA was quantified as described in Sect. 2.6.5.

2.6.2 DNA extraction from agarose gel

PCR fragments, which were used for cloning or labelling as probes, were gel extracted using QIAquick Gel Extract Kit (Qiagen). The extraction procedures recommended by the supplier were followed. The concentration of extracted DNA was estimated as in Sect. 2.6.5.

2.6.3 DNA extraction from polyacrylamide gel

For cloning AFLP fragments, the QIAEXII Polyacrylamide Gel Extraction Kit (Qiagen) was used to extract DNA from polyacrylamide gel. The DNA fragment were excised from the polyacrylamide gel and crushed in 50 µl diffusion buffer followed by incubation at 50 °C for

Diffusion buffer

0.5 M NH_4Ac
10 mM $MgAc_2$
1 mM EDTA pH 8.0
0.1 % SDS

30 min or overnight. After centrifugation for 3 min, the supernatant was carefully transferred to a new Eppendorf tube to which 150 µl of Buffer QX1 was added. The 10 µl of QIAEXII were added subsequently after resuspending. DNA bound to the QIAEXII particles was eluted in 20 µl Buffer EB (10 mM Tris-HCl, pH 8.5) after washing with PE buffer and air-drying. 2-5 µl of the eluate was taken as template for PCR reaction in 30 µl volume.

2.6.4 Plasmid DNA extraction

5 ml overnight-cultured bacteria were harvested by centrifugation and plasmid DNA was extracted using QIAprep® Spin Miniprep Kit following the supplier's protocol. The concentration of plasmid DNA was measured as in Sect. 2.6.5.

2.6.5 Concentration measurement of DNA

The quality and quantity of DNA were measured by comparison of band-intensity on ethidium bromide stained agarose gels with a DNA molecular weight standard, or alternatively, by the absorbance at 260 nm.

2.7 Cloning of PCR and AFLP fragments

2.7.1 Ligation of fragments in pGEM® -T

A-tailing of PCR product

The pGEM®-T needs an A-tailed PCR product for ligation. When using fresh PCR products, tailing was not necessary as *Taq* DNA polymerase has the function of adding adenosine at the end. Otherwise, an A-tailing was done before ligation.

Tailing reaction

1-2 µl purified PCR product
1 µl 10× PCR buffer for *Taq* DNA polymerase
0.5 µl 50 mM $MgCl_2$
1 µl 2 mM dATP
1 µl *Taq* DNA polymerase (5 U/µl)
add deionized water to 10 ml
incubate at 70 °C for 15-30 min

Insert : vector molar ratios

A molar ratio 3:1 of insert : vector was adopted to calculate the amount of PCR product for

the ligation reaction according to the following formula:
PCR product (ng) = 3 × (50 ng vector) × (size of PCR product in kb)/3.0 kb vector
Ligation was done according to the supplier's instruction.

Ligation reaction

5 µl 2× rapid ligation buffer for T4 DNA ligase 1 µl pGEM®-T vector (50 ng/µl) 3 µl PCR product (concentration optimised) 1 µl T4 DNA ligase (3 U/µl)	Incubate the reaction overnight at 4 °C before transformation.

2.7.2 Transformation of *E. coli*

Preparation of competent host cells

Glycerol stock of *E. coli* strain TG2 was streaked on LB medium and incubated at 37 °C overnight. A single colony was picked and cultured overnight in 5 ml of LB liquid medium followed by subculture of 2 ml in 500ml LB in a 2.5-liter flask. When optical density (OD) was in the range of 0.5-0.6 OD, cells were harvested by centrifugation at 5000 rpm, 4 °C for 10 min. Cells were washed 3 times by gently resuspending and centrifuging in sterile ice-cold water. Cells were then washed once using 10 ml 10 % glycerol. The cell pellet was gently resuspended in 2 ml of 10 % glycerol. The cell suspension was divided into 100 µl-aliquot, and frozen in liquid nitrogen and stored at –70 °C.

Transformation

For each transformation, 30 µl of competent cells of *E. coli* strain TG2, thawed on ice, were mixed with 1.6 µl of ligation mixture and transferred to the electroporation cuvette avoiding bubbles. Electroporation was done at 1.7 kV on an electroporator (BioRad). The cells were removed from the chamber in 300 µl SOC medium, transferred to a 5 ml culture tube and immediately incubated at 37 °C for 40-90 min. 100 µl of bacteria was plated on the white and blue selective LB media containing ampicillin (100 µg/ml), IPTG (8 µg/ml) and X-Gal (40 µg/ml). Petri-dishes were incubated at 37 °C for 14-20 h.

Screening

Single white colonies was picked out with a sterile tip and dipped in a PCR master mix to amplify the target insert of plasmid using appropriate primer sets. In parallel, the single colony was also streaked on an LB plate. Once the correct PCR product was obtained, the corresponding colony was propagated for plasmid DNA isolation and sequencing.

2.8 DNA sequencing and sequence analysis

PCR products were separated on a 1.5 % agarose gel and single bands were cut from the gel. DNA was recovered using the QIAquick Gel Extraction kit (Qiagen). Alternatively, PCR fragments were cloned into pGEM®-T vector by transforming *E. coli* strain TG2. Plasmid DNA was extracted using QIAprep Spin Mini-prep Kit (Qiagen) (see Sect. 2.6.4). The ABI PRISM Dye Terminator cycle Sequencing Ready Reaction Kit was used (Applied Biosystems) for sequencing on an Applied Biosystems 377 DNA Sequencer (Applied Biosystems).

Sequence analyses were performed using sequence analysis software package GCG (Wisconsin University, Version 9.1, UNIX, September 1997). Updated versions of the sequence databases GenBank and EMBL were searched for the sequences of functional genes available in the databases. For sequence comparison, the *Bestfit* or *Pileup* programs were used.

2.9 Restriction fragment length polymorphism (RFLP) analysis

2.9.1 Genomic DNA restriction and purification

5 µg genomic DNA were digested overnight by 20 U *Taq*I or *Alu*I in buffer B at 65°C or buffer A at 37°C. Digested DNA was mixed with 2 volumes of ice-cold 100 % ethanol and 1/20 volume of 4 M NH_4Ac, precipitated and collected by centrifugation at 14000 rpm for 30 min. The pellet was washed with 70 % ethanol and air-dried, followed by adding 5 µl formamide loading buffer. The pellet was resuspended by vortexing, denatured at 95 °C for 5 min and chilled on ice before loading.

2.9.2 Fragment separation by electrophoresis

DNA fragments were separated on a 4 % denaturing polyacrylamide gel as described by Kreitman and Aquade (1986) and modified by Gebhardt *et al*. (1989).

Gel mix

62.4 g urea
13 ml 40 % polyacrylamide/ bisacrylamide (20:1)
13 ml 10 × TBE
add deionized water to130 ml

Gel mix was filtered through 0.2 µm filter and degassed under vacuum for 5 min. After adding 200 µl 25 % APS (ammonium persulfate) and 150 µl TEMED and gentle mixing, the gel was poured. The gel was 30 cm wide, 40 cm high and 0.1 cm thick with loading slots of 0.4 cm in width. Gel was polymerized for at least two hours at room temperature before loading the samples. The

electrophoresis was run in 1 × TBE (1.8 liter) (see Sect. 2.5) as running buffer. Power was set first to 15 W for 15 min followed by a 5-6 h run at a constant power of 50 W for until the xylene cyanol marker reached the bottom of the gel.

Formamide loading buffer

94 % formamide
0.05 % xylene cyanol
0.05 % bromo phenol blue
10 mM EDTA pH 7.2

2.9.3 Electro-blotting of DNA fragments

Electro-transfer of DNA fragments onto Nylon-membrane (Pall, Biodyne) was performed in 23 liters of 0.4 × TPE buffer in a home-made blotting chamber (45 cm long and 35 cm wide) for 1 hour at 35 V (5 V/cm). DNA was covalently cross-linked to the membrane by drying at 80°C for 30 min. The membrane was then used for hybridisation or stored in 2 × SSC solution at 4 °C.

2.9.4 Radioactive labelling of DNA probes

5 × OLB buffer

0.1 mM dATP, dCTP, dGTP, dTTP
1.0 M HEPES, pH 6.6
2.0 mg/ml Hexadesoxyribonucleotide pdN6 (Pharmacia)
0.2 M Tris-HCl, pH 8.0
3.7×10^{-5} M $MgCl_2$
0.35 % (v/v) β-Mercaptoethanol

Sephadex buffer

10 mM Tris-HCl, pH 7.5
1 mM EDTA
0.3 mM NaCl
0.1 % SDS

filter sterilise or autoclave

Sephadex suspension

100 ml Sephadex buffer
2 g Sephadex-G50

autoclave

Random primer labelling reaction (20 µl)

4 µl 5 × OLB
1 µl Klenow-Polymerase (2 U/µl)
3 µl of [α^{32}P]-dCTP (10 µCi/µl)
12 µl (50-100 ng) denatured DNA

50-100 ng of gel-purified DNA was denatured in 12 µl volume at 95°C for 5 min and chilled immediately on ice. The random primer labelling method (Feinberg and Vogelstein 1983, 1984) was used to label the probe DNA with [α^{32}P]-dCTP (10 µCi/µl) in a total reaction volume of 20 µl.

Leave the labelling reaction at room temperature overnight or at 37°C for 1h. The separation of labelled DNA-fragments from unincorporated nucleotides was done using a 1 ml-Sephadex G-50 column. The 1 ml syringe was filled with sterile glass wool at the bottom and G-50 Sephadex equilibrated in buffer (G50-Sephadex suspension). The labelling reaction was mixed with 300 µl Sephadex suspension and eluted through the column. A second elution

with the same amount of Sephadex suspension was performed and the second elution was collected. Labelling efficiency was checked using the radioactive counter. The DNA probe was mixed with 80 µl hering sperm DNA (5 mg/ml) and denatured at 95°C for 5 min.

2.9.5 Hybridisation

Southern blot hybridization was performed in glass tubes (30 cm x 3.5 cm in diameter). Up to 3 membranes were hybridised in one tube. The membranes were stacked on top of each other, then rolled up with a plastic stick and unrolled around the inner wall of the tube. Tubes were tightly closed with plastic screw caps with sealing rubber inside. 10 ml 6 × SSC per membrane and 40 µg/ml denatured hering sperm DNA were added to the tube for pre-hybridisation and hybridisation. For pre-hybridization the tubes were incubated at 65 °C, rotating around the long axis in a hybridisation oven (Bachofer, Reutlingen, Germany) for at least 4 hours. After adding the denatured probe, the hybridisation was performed overnight under the same conditions as pre-hybridisation.

After hybridisation the filters were washed at 65 °C in the hybridisation oven in the same tube three times 15 min each, using 50 ml of pre-heated washing solution (1 × SSC, 0.1 % SDS). Then the filters were taken out from the tube and washed two times in a tank containing sodium phosphate buffer at room temperature for at least 30 min. The filters were wrapped in thin plastic foil (Saran film) and placed in cassettes with intensifying Trimax-screen. Kodak X-OMAT AR5 film was placed on the filter and exposed at –70°C for about 10 days before developing. Alternatively, filters were exposed on Fuji phosphoimage screens for 5 days. Fragment patterns were visualised using a Fuji BAS-2000 Phosphoimager (Fuji Photo Film Company Ltd., Japan).

6 × SSC

55.2g/l NaCl
26.4g/l Sodium citrate
(pH7.0)

Hybridising solution

7 % SDS
0.12 M Na_2HPO_4
1 mM EDTA
40 µg/ml denatured hering sperm DNA,
(pH 7.0)

Washing buffer

1 × SSC
0.1 % SDS

Sodium phosphate buffer

5 mM Na_2HPO_4
1 mM EDTA
0.2 % SDS, pH7.0

2.10 Sequence-tagged sites (STS) analysis

2.10.1 Exploration of functional gene sequences in databases

GenBank and EMBL databases were explored for sequence information of genes involved in carbohydrate metabolism including the Calvin cycle, the oxidative pentose phosphate

pathway, the citric acid cycle, glycolysis, sucrose and starch metabolism and intermediate transport. The exploration of functional genes was also expanded to other plant species such as tomato, tobacco and *Arabidopsis* in case when gene sequences are not available in potato.

2.10.2 Nomenclature of the functional genes

The rules of CPGN (the Commission on Plant Gene Nomenclature) were followed for gene nomenclature in potato. Genetic loci and corresponding genes were given italicised three-letter designations, starting with one upper case letter. The three letters reflect a particular property of gene function. Different loci detected by the same gene were distinguished by the assignment of an additional letter a, b or c. Gene products were designated in non-italicised capital letters. When the same three-letter code has been assigned to genes in different species, the species two-letter code *'St'* for potato, *'Le'* for tomato and *'At'* for Arabidopsis are added to gene names.

2.10.3 Polymerase chain reaction (PCR)

Primer design

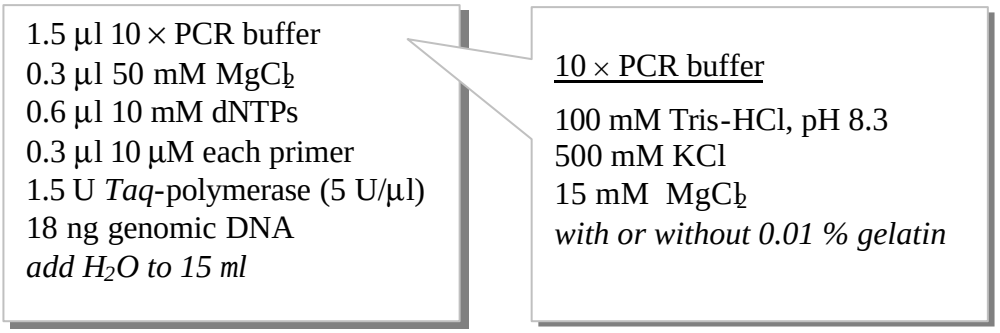
The following criteria were followed to design STS primers. First, primer design should avoid the intron-exon junctions since most of the available sequences in the public databases are cDNAs. Second, for detection of polymorphism, primer positions are ideally chosen to frame one or two introns since these regions are potentially variable at the intraspecific level (Weining and Langridge 1991; Tragoorung *et al*. 1992; Konieczny and Ausubel 1993; Cote-Real *et al*. 1994). Finally, primers should have good characteristics for amplification with optimal annealing temperature of 55-60 °C and primer length between 22 and 24 bases.

In cases when gene sequences were not available in potato, tomato sequences were employed instead. When neither a potato nor a tomato sequence was available, degenerate primers based on sequence conserved between genes from other plants were designed. Consequently, these primers were composed of a mixture of several different oligonucleotides supposedly in equivalent proportion.

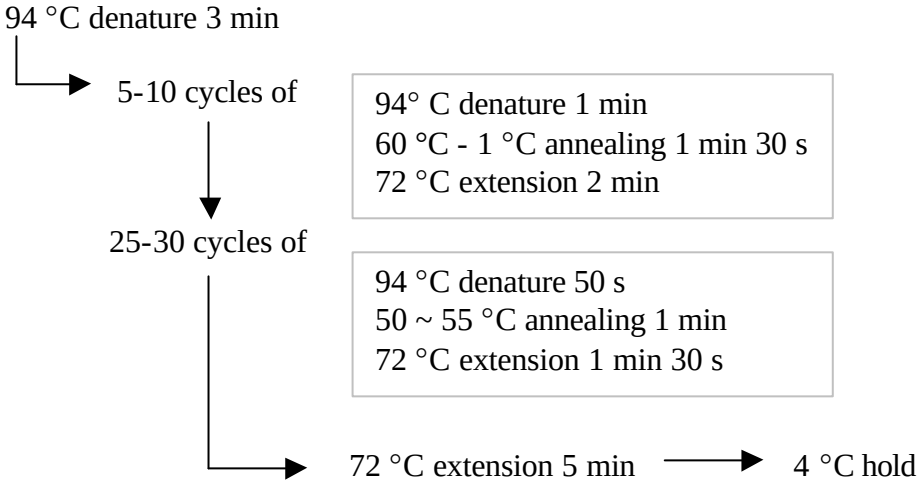
PCR reaction conditions

PCR reaction was performed in 15-100 µl reaction volume depending on the amount of PCR product needed. Mostly, PCR reactions were set up in a final volume of 15 µl. The reactions mix was capped with a drop of mineral oil that was omitted when reactions were performed in a heating-lid thermo-cycler (Biometra, Göttingen). A 'touchdown' amplification protocol was employed (Kresovich *et al*. 1995) with the following conditions:

PCR reaction (15 ml)



PCR conditions



2.10.4 Identification of sequence characterized amplified regions (SCAR)

To discover SCAR polymorphisms, 5-10 individuals of populations K31 and LH were randomly selected for PCR amplification. PCR products were analysed by electrophoresis on 1.5 % - 2 % agarose gel in 1× TAE. 1-kb ladder was used as size marker. DNA fragments were visualised by ethidium bromide staining.

2.10.5 Identification of cleaved amplified polymorphic sequences (CAPS)

Monomorphic PCR products were digested for CAPS with a range of restriction endonucleases. These restriction enzymes were 4-bp cutter including *Alu*I, *Rsa*I, *Taq*I, *Hpa*II, *Dde*I, *Hae*III, *Mse*I, and *Mbo*I. Two units of restriction enzyme, mixed with the reaction buffer, were added to 15 µl of the PCR reaction to final volume of 20 µl. The digests were incubated at least 3 h or overnight at the recommended temperature. CAPS polymorphisms were analysed by agarose gel electrophoresis as described above.

2.11 Generation of segregation data and linkage analysis

When the approaches as outlined in Sect. 2.10.4 and 2.10.5 detected a polymorphism, parents P3, P38 and 90-150 progeny of populations K31 and 49 progeny and parental lines T710 and 45C3 of population LH were analysed by PCR. Fragments were scored as 0, 1 and 2 for absence, presence and missing value, respectively. The segregation data was then added to the existing RFLP framework data sets (Schäfer-Pregl *et al*. 1998). Recombination frequencies were computed using the software package MAPRF (version 2.1, E. Ritter). The recombination frequency between any two markers was displayed in the MAPDIC sub-program. Linkage of a new marker to an existing linkage group was identified using the FIND command. The PATTERN command was used to check the recombinant genotypes for scoring errors (singletons). The genetic distance between marker pairs is measured by the recombination frequency. The linear order of the markers in a linkage group was determined based on pairwise recombination frequencies in two-, three-, or multiple-point estimates (Ritter *et al*. 1990). The recombination values were converted into map distances (cM) by Kosambi's mapping function (Kosambi 1944).

2.12 Detection of single nucleotide polymorphisms (SNPs)

Gene *Stp23* was selected as a case study for detecting SNPs in functional genes. Genomic DNA from 14 potato lines (see Sect. 2.2) was used to amplify the homologous segment of gene *Stp23* by PCR. PCR products were bi-directionally sequenced. The PCR products of three lines P3, P38 and T710 were additionally cloned into pGEM®-T vector prior to sequencing. Electropherograms of nucleotide sequence data were visually examined for alternative nucleotides (SNPs) at specific positions in individual genotypes. Sequence data of different lines were assembled by *Pileup* program of GCG software package (see Sect. 2.3). Each vertical slice of the resulting assembly was examined for nucleotide variation. Any such slice was considered a candidate SNP.

2.13 Amplified fragment length polymorphisms (AFLP)

2.13.1 Radioisotope-labelled amplified fragment length polymorphism (rAFLP) analysis

DNA digestion and ligation of adaptors

35-250 ng of genomic DNA was incubated for 1 h at 37 °C with 5 units of *Eco*RI and 5 units of *Mse*I, 4 µl of 10 × RL buffer in a 40 µl reaction volume.

Restriction reaction (40 µl)

4 µl 10 × RL buffer
2 µl *Eco*RI (10 U/µl)
2 µl *Mse*I (10 U/µl)
35-250 ng genomic DNA
add deionised water to 40 ml

10 × RL buffer

100 mM Tris-HCl
100 mM MgAc
500mM KAc

Adaptors were prepared by mixing equal-molar amounts of both stands, denatured at 95 °C for 5 min and renatured at room temperature over 10 min.

Ligation reaction (50 µl)

40 µl restriction mixture
1 µl *Eco*RI-adaptors (5 pM/µl)
1 µl *Mse*I-adaptors (50 pM/µl)
1 µl 10 mM ATP
1 µl 10 × RL buffer
1 unit of T4 DNA ligase

10 µl of a solution containing 5 pMol of *Eco*RI-adaptors, 50 pMol of *Mse*I-adaptors, 1 µl of 10 mM ATP, 1 µl of 10 × RL buffer, and 1 unit of T4 DNA ligase was added to the restriction solution, and the incubation was continued for 3 h at 37 °C.

After ligation, the reaction mixture was diluted 1-10 fold with TE10/0.1 (see Sect. 2.5) buffer depending upon the amount of DNA used for restriction. The reference dilution was 10-fold when 250 ng genomic DNA were used for the restriction and ligation. Reactions with different amounts of DNA were diluted based on the their proportion of DNA to that of the reference dilution. The calibrated restriction and ligation mixtures were stored at –20 °C.

Preselective amplification

Preselective amplification reaction (20 µl)

2 µl 10 × PCR buffer
0.4 µl 50 mM $MgCl_2$
0.8 µl 10 mM dNTPs
0.4 µl 10 µM each primer
0.4 µl *Taq*-polymerase (5 U/µl)
4 µl template DNA
add H_2O to 20 ml

Reaction conditions

The pre-amplification reactions were performed for 20 cycles with the following cycle profile: a 30 s DNA denaturation step at 94°C, a 30 s annealing step at 56 °C, and a 1 min extension step at 72°C. The amplification products were diluted 20-fold with TE10/0.1 buffer before proceeding to selective amplification.

Labelling of primers and size marker with [γ^{33}P]-ATP

Primer labelling reaction (12.5µl reaction volume for 25 selective amplification reactions)

The labelling reaction mix was incubated at 37 °C for 45 min, and then stopped by incubating at 75 °C for 10 min.

1.3 µl 10 × T4 PNK buffer
0.4 µl T4 polynucleotide kinase (PNK) (6 U/µl)
2.5 µl *Eco*RI-primer (10 pM/µl)
2 µl [γ^{33} P]-ATP (10 µCi/µl)
add H_2O to 13ml

10 × T4 PNK buffer
250 mM Tris-HCl pH 7.5
100 mM $MgCl_2$
50 mM DTT
5 mM Spermidine-HCl

Size marker labelling

30-330 bp AFLP DNA ladder, not dephosphorylated, was radioactively labelled either by the exchange reaction or as in previous part after dephosphorylation.

Labelling by exchange reaction

Set up reaction mix in 0.2 ml PCR tube:

1 µl 5 × Exchange reaction buffer
2 µl 30-330bp AFLP DNA ladder
1 µl [γ^{33} P]-ATP (10 µCi/µl)
1 µl T4 polynucleotide kinase (10 U/µl).

5 × Exchange reaction buffer
250 mM imidazole (pH 6.4)
60 mM $MgCl_2$
5 mM 2-mercaptoethanol
350 µM ADP

Collect solution by brief centrifugation, incubation for 10 min at 37 °C and stop the reaction by heating the tube 15 min at 65 °C.

Before loading on the gel, add equal volume of loading buffer to AFLP samples.

Dephosphorylation of marker DNA (40 µl)

5 µl 30-330 bp AFLP DNA ladder (Life Tech.)
4 µl 10 × SAP buffer
2 µl Shrimp alkaline phosphatase (1 U/µl)
29 µl H_2O

Incubate at 37 °C for 45 min, stop the reaction by addition of 0.5 µl 0.5 M EDTA (pH 8.0) and incubate at 75 °C for 10 min. The deposphorylated DNA marker can be use directly for labelling without precipitation and purification.

Labelling the dephosphorylated AFLP DNA ladder

Set up the labelling reaction in a 0.2 ml PCR tube (10 µl). Incubate for 45 min at 37 °C, followed by 10 min at 70 °C to stop the reaction. 1-2 µl was taken and added equal volume of AFLP loading buffer before gel loading.

7.5 µl dephosphorylated AFLP DNA ladder
1 µl 10 × T4 PNK buffer
1 µl [γ^{33} P]-ATP (10 µCi/µl)
0.5 µl T4 PNK (6 U/µl)

Selective amplification

Selective amplification was carried out with two oligonucleotide primers to which two more selective nucleotides had been added at the 3' end. One of the two primers, *Eco*RI-primer, was end-labelled. PCR was performed in 20 µl reaction volume.

Selective amplification reaction (20 µl)

2 µl 10 × PCR buffer
0.4 µl 50 mM $MgCl_2$
0.4 µl 10 mM dNTPs
0.6 µl 50 ng/µl non-labelled primer
0.5 µl labelled primer
0.2 µl *Taq*-polymerase (5 U/µl)
5 µl diluted preamplification products
add H_2O to 20 ml

Selective amplification reaction conditions

The AFLP reactions with primers having three selective nucleotides were performed for 36 cycles with the following cycle profile: a 30 s DNA denaturing step at 94 °C, a 30 s annealing step, and a 1 min extension step at 72 °C. The annealing temperature was 65 °C in the first cycle, subsequently reduced by 0.7 °C per cycle for the next 12 cycles, finally stabilising at 56 °C for the remaining 23 cycles. All amplification reactions were carried out in a same thermocycler.

Denaturing polyacrylamide gel analysis of AFLP

The AFLP selective amplification products were mixed with an equal volume (20 µl) of formamide loading buffer.

Formamide loading buffer for rAFLP

98 % formamide
10 mM EDTA pH 8.0
0.025 % bromophenol blue
0.025 % xylene cyanol

The samples were denatured for 3 min at 95 °C, and then quickly chilled on iced. 4 µl of each sample were loaded on a 5 % denaturing polyacrylamide gel.

The rAFLP gel mix

75 ml 5 % acrylamide/ bisacrylamide (20:1) solution
50 µl TEMED
750 µl 10 % APS

5 % acrylamide/ bisacrylamide (20:1) solution (1 liter)

250 ml acrylamide-bisacrylamide (20:1)
450 g urea
50 ml 10 × TBE for AFLP
add distilled water to 1 litre
filter sterilised and stored at 4 °C

The 5 % acrylamide / bisacrylamide (20:1) solution was degassed 5 min. Then 750 µl

10 % APS (ammonium persulfate) and 50 µl TEMED were added and gently mixed. Gels were cast using a 38 × 50 cm gel glass plate apparatus (BioRad). The gels were fixed onto one of two glass plates, one had been treated with sticking solution (0.3 % acetic acid and 0.015 % ?-methacryloxypropyltrimethoxysilane in ethanol) whereas the other one with repellent solution (Acrylease™, Stratagene, Heidelberg, Germany). The gel was polymerized for at least two hours or overnight at room temperature. A pre-run was performed for 30 min in pre-heated 0.5 × TBE buffer before loading the gel. The gel was run at a constant power of 58 W for 2-3 h until the xylene cyanol marker reached the bottom of the gel.

After electrophoresis, the gel on the glass plate was fixed in 10 % acetic acid for 30 min, followed by rinsing with distilled water. After drying in an oven at 80 °C for 1.5 h, the glass plate was wrapped with Saran film and exposed to a Kodak X-OMAT AR5 film at room temperature for 1-2 days before developing.

2.13.2 Fluorescent dye-labelled amplified fragment length polymorphism (fAFLP) analysis

Table 2-1 Adaptor and primer sequences and fluorescent labelling dye used for fAFLP analysis

Primer/adaptor	Labelling dye	Sequence (5´-3´)
*Eco*RI-adaptor		CTCGTAGACTGCGTACC CATCTGACGCATGGTTAA
*Eco*RI+1 primer		GACTGCGTACCAATTC-a (=E)
*Eco*RI+3 primers	FAM (blue)	E-aca, E-act
	JOE (green)	E-aag, E-acg, E-agg
	TAMRA (yellow)	E-agc, E-acc
*Mse*I-adaptor		GACGATGAGTCCTGAG TACTCAGGACTCAT
*Mse*I+1 primer		GATGAGTCCTGAGTAA-c (=M)
*Mse*I+3 primers		M-caa, M-cac, M-cag, M-cat, M-cta, M-ctc, Mctg, M-ctt
*Hind*IIII-adaptor		CTCGTAGACTGCGTACC CATCTGACGCATGGTCGA
*Hind*III+1 primer		AGACTGCGTACCAGCTT-a (=H)
*Hind*III+3 primer	FAM (blue)	H-act
	JOE (green)	H-agt, H-aga
	TAMRA (yellow)	H-ata
*Taq*I-adaptor		GACGATTGAGTCCTGAC TAACTCAGGACTGGC
*Taq*I+1 primer		ATTGAGTCCTGACCGA-a (=T) ATTGAGTCCTGACCGA-g
*Taq*I+3 primer		T-agt, T-atg, T-gta, T-gat

For fAFLP genotyping with *Eco*RI/*Mse*I primer sets, reagents including *Eco*RI adaptor pairs, *Mse*I adaptors, preselective and selective amplification primers and AFLP Core Mix were obtained from Applied Biosystems (ABI). For screening with other primer sets such as *Eco*RI/*Hind*III, *Eco*RI/*Taq*I and *Hind*III/*Taq*I, primers and corresponding adaptors were designed and custom synthesised by MWG-Biotech (Munich). The sequences of adaptors and primers are shown in Table 2-1 (on page 39). The fAFLP procedures followed the supplier's instructions (PE Applied Biosystems).

Restriction and ligation reaction

Annealing of the adaptors (see Sect. 2.13.1)

Restriction enzyme master mix (for 50 DNA samples in 50 µl)

5 µl 10 × T4 DNA ligase buffer with ATP
5 µl 0.5 M NaCl
2.5 µl 1 mg/ml BSA
50 U *Mse*I
250 U *Eco*RI
50 U T4 DNA ligase (400 U/µl)
add sterile deionised water to 50 ml

1 × T4 DNA ligase buffer with ATP
50 mM Tris-HCl (pH 7.8)
10 mM $MgCl_2$
10 mM dithiothreitol
1 mM ATP
25 µg/ml bovine serum albumin

Reaction mix (11 µl)

5.5 µl (35 ng-500 ng) genomic DNA
1 µl 10 × T4 DNA ligase buffer with ATP
1 µl 0.5 M NaCl
0.5 µl 1 mg/ml BSA
1 µl *Eco*RI adaptor pair
1 µl *Mse*I adaptor pair
1 µl restriction enzyme master mix

Mix thoroughly and spin down to the bottom of the tube. Incubate at 37 °C for 3 h using a thermal cycler with a heated lid to prevent *Eco*RI* (star) activity.

Dilution of the restriction-ligation samples

Dilution of restriction-ligation samples depended on the amount of DNA used. When 500 ng genomic DNA were used, the sample was diluted 20-fold by adding 189 µl of $TE_{10/0.1}$. Otherwise, dilution was adjusted described in Sect. 2.13.1.

Amplification of target sequences

Preselective amplification reaction

4 µl diluted restriction-ligation DNA 1 µl AFLP preselective primer pairs 15 µl AFLP Core Mix*

* AFLP Core Mix was a reagent mixture including AmpliTaq enzyme, buffer and dNTPs that was provided by ABI.

Preselective amplification reaction conditions

The preselective amplification reactions were performed with the following PCR profile: first incubate at 72 °C for 2 min, then 20 cycles of a 1 s at 94 °C, a 30 s at 56 °C, 2 min at 72 °C, and finally, incubating at 60 °C for 30 min.

Dilution of preselective amplification products

The preselective amplification reaction products were diluted 20-fold with $TE_{10/0.1}$ before proceeding to the selective amplification.

Selective amplification reaction

1.5 µl diluted preselective amplification product 0.5 µl 5 µM *Mse*I Primer 0.5 µl 1 µM dye-labelled *Eco*RI primer* 7.5 µl AFLP Core Mix

* the *Eco*RI primers were either labelled with 5-carboxy-fluorescein (5-FAM, blue), 2',7'-dimethoxy-4',5'-dichloro-6-carboxy-fluorescein (JOE, green) or N,N,N',N'-tetramethyl-6-carboxy-rhodamin (TAMRA, yellow). The internal size standard was Genescan-500 ROX length standard (PE Applied Biosystems) labelled with 6-carboxy-X-rhodamin (ROX, red).

Selective amplification reaction conditions

First cycle: 94 °C for 2 min, 65 °C for 30 s, 72 °C for 2 min, next 8 cycles: 94 °C for 1 s, 64 °C for 30 s (with 1 °C decreased for each cycle) and 72 °C for 2 min, followed by 23 cycles of 94 °C for 1 s, 56 °C for 30 s and 72 °C for 2 min with a final step of incubation at 60 °C for 30 min.

Samples preparation

<u>Loading dye mix (for 100 samples)</u> 170 µl formamide 40 µl blue dye (loading buffer, ABI) 30 µl size standard of GS-500 ROX

2 µl selective amplification product of each sample was mixed with 2.4 µl aliquot of loading dye denatured at 95 °C for 2 min, then immediately chilled on ice until ready for loading.

Gel electrophoresis on 377 DNA Sequencer

Plate preparation

Wash the plates two times with 2N NaOH and Alconox™ alternately and rinse with distilled water, stand the plates up right and allow to air dry. Similarly, wash, rinse and dry the comb and the spacers.

Gel preparation

Gel mix for fAFLP

21 g urea (Merck)
8.4 ml 30 % acrylamide/bisacrylamide (29:1) solution
6 ml 10 × TBE (Roth)
20 ml H_2O (Merck)

Stir the gel mix solution until urea is completely dissolved. Filter the solution through a 0.2 µm cellulose nitrate filter and degas for 5 min. Add 350 µl 10 % APS and 15 µl TEMED, swirl gently to mix and cast the gel by injecting the solution with a syringe in the space between the plates. Insert the comb avoiding to trap bubbles at the interface. Allow gel to polymerise for 2 h before use.

Instrument set up

Clean read region and comb area of the plates with Kimwipes™ and distilled water, then place the gel cassette against the heat transfer plate of the instrument. Fill the upper buffer chamber with 580-600 ml 1 × TBE (Roth) and check for leaks. Fill the lower buffer chamber with 1 × TBE until the bottom of the glass plates is covered. Place the front heat-transfer plate on the front of the cassette. Secure it with and attach the quick-connect water lines and ground cable. Plug in the electrode lead to the jacks extending from the buffer chambers.

Software set up

Restart the Macintosh computer and open the ABI PRISM™ 377 collection. Choose genetic analysis program and create a run file. Set parameters as follows:

Plate Check Module: plate check A
PreRun module:
GSPR 36A-2400 with constant voltage of 1100V and gel temperature of 51 °C
Run Module:
GS Run 36A-2400 with constant voltage of 3000V and gel temperature of 51 °C
Collection time: 4h
Gel's Matrix File:
GS Set A for detection of fluorescent dye: FAM, JOE, TAMRA and ROX
Well-to-Read distance: 36 cm
Lanes: 50, 66 or 96 depending on the number of samples to be analysed
Run Mode: XL Scan

Before loading the gel, pre-run the gel to make sure the electrophoresis system is working properly and check for temperature equilibration. Pre-run the gel for 15 min under the conditions defined in the run file.

Sample sheet file

Choose 'new' from the file menu, a sample sheet will appear by clicking the GeneScan® Run icon. Fill out the form with relevant information of samples and save it.

Loading the samples and starting the run

Before loading the gel, click 'Pause' button to arrest the instrument. Pausing stops electrophoresis but maintains the temperature of the gel. 1.5 µl of each sample was loaded in an order identical to that of the sample sheet. After loading, click the 'Cancel' button to stop the pre-run and click the 'Run' button to start the run under the conditions defined in Run File.

Data collection and fragment sizing by ABI GeneScanÒ

Automated AFLP fragment analysis was performed with GeneScan® analysis software version 2.1 (Perkin-Elmer/ABI). In automated genotyping on a 377 sequencer, the fluorescence labelled DNA fragments are separated on the polyacrylamide gel. At the bottom of the gel, a laser excites the fluorescent dyes when the fragments pass, and detectors collect the emission intensities at specific wavelengths as defined in GS Set A. The laser and detectors scan the bottom of the gel continuously during electrophoresis in order to build a gel image in which each lane has a colour pattern of peaks, each peak corresponding to a fragments of particular length. The ABI GeneScan® software, running on a Macintosh® computer, collects each fluorescent signal and then assigns a size to each fragment.

The analysis parameter

Analysis range: full range
Size call range: all sizes
Peak detection: all peaks higher than 50 were taken as real fAFLP fragments.

GS-500 size standard

Select a sample file containing GS-500 size standard and assign the known size in bp to each fragments of the size standard in the size standard dialog box.

Samples Analysis

Open the 'project' window containing the data, select the size standard, the analysis parameters and colours for all samples and the internal size standard. Click the 'analyse'

button for automated sizing of fragments. Before proceeding to allele scoring, each sample file was checked to be sure that the sizes of the size standard correspond well with what has been defined.

Allele scoring by ABI GenoTyper™

In this study Genotyper™ analysis software version 2.0 (PE Applied Biosystems) was used for creation of data files. GenoTyper™ converts data from GeneScan® files into the Excel format required by downstream applications. The following steps are involved in the processes:

Importing result files from GeneScanÒ files

Creating categories

The categories define the peaks of interest that need to be labelled. In case of AFLP, where no information of the AFLP fragments was available, categories were defined which took all fragments ranging from 50 bp to 500 bp into account. One base pair corresponds, therefore, to one category with a tolerance of $\pm$ 0.49 bp.

Labelling peaks

Peak labelling was done either quantitatively as peak height, or qualitatively as presence versus absence.

Making tables

After auto-labelling, data tables for exporting were made which contained sample info, categories and other relevant information. These data can be exported in Excel format for QTL analysis.

2.14 Evaluation of phenotypic traits

Phenotypic data were evaluated in field trials at two locations (Gransebieth and Windeby) over three years (1997, 1998, and 1999) by the potato breeding company SaKa-Ragis Pflanzenzucht GbR (Dr. Josef Strahwald). A short description is included here because the phenotypic data were an essential part of the QTL analysis.

Trait evaluation was carried out on 150-200 individuals of Z3 in trials 1997 and 1998. In trial 1999, evaluation was performed on 50 individuals, which were obtained by selecting 4-6 individuals with high and low tuber starch content in pairwise from each of 9 categories for foliage maturity (1-9 scale). Phenotypic trait evaluated varied in different trials, however, present study concentrates on the analysis for the following traits.

Starch content

Tuber starch content in percent was determined by measuring specific gravity (weight in air – weight in water) using a starch balance (Meku, Wennigsen, Germany) based on the method of Lunden (1956).

Reducing sugars

Reducing sugar content was measured by a Picric Acid Test. The degree of darkening of a solution of potassium picrate is a measure for the reducing sugar content of tubers. Picric acid reacts with reducing sugars to produce a red colour. In solutions containing excess picric acid, the red colour will combine with the yellow colour of the picric acid to produce a series of shades from yellow through orange to red. Reducing sugar content was estimated by comparing the colour with a series of colour standards which have been calibrated in terms of reducing sugar (see below).

CTC[a]	1	2	3	4	5	6	7	8	9
RSC[b]	0.059	0.12	0.20	0.28	0.38	0.49	0.67	0.95	1.35

[a] CTC=Colour Test Classification; [b] RSC=Reducing Sugar Content in %

Foliage Earliness (Maturity)

Foliage earliness was scored using a scale of 1 to 9 (1=very early maturing, 9=very late maturing) at an appropriate growth stage of the plants.

2.15 QTL analysis

QTL analysis was carried out in collaboration with Dr. Ralf Schäfer-Pregl (MPIZ).

Several statistical methods can be used for the identification of QTLs depending upon the molecular marker types and scoring methods. In the present study, the statistical programs used for the QTL analysis include the t-test or one way ANOVA (analysis of variance) and GLM (general linear models). All analyses were performed with programs written using SAS software (SAS Institute Inc., 1990).

t-test

A t-test was applied to the fAFLP data which were divided into two genotype classes such as presence and absence of a DNA fragment. The means of the two classes are tested for

significant differences by estimating the probability of no difference between the means compared.

ANOVA

One-way ANOVA is an alternative to the t-test. This procedures tests the significance of the difference between the means of two subgroups by estimating the F value, the proportion of the variance between the two subgroups to the variance within each group, from which a *P* value or a LOD (logarithm of odds) value are derived. This procedure also gives the R^2 value, indicating the proportion of the total variance that is explained at the marker locus considered.

GLM

In case when multiple alleles are informative at given locus and can be distinguished in a segregating population, GLM was applied to the QTL analysis by examining the variance among the phenotypic trait means of multiple marker-genotype classes. Using this method, the amount of variance explained by allelic differences at a marker locus (R^2) can also be calculated.

Regression analysis

Regression analysis was applied to quantitative fAFLP data. This analysis reveals the correlation between the peak height and the phenotypic values. The square of the correlation coefficient reflects the proportion of the variance explained by regression to the total variance.

Non-parametric test

For the category phenotypic data, the Mann-Whitney U-test and the Kruskall-Wallis test were employed for the association analysis. The former is the non-parametric equivalent to the t-test, while the latter one is the non-parametric counterpart of GLM.

Thresholds for declaring a QTL present

A marker-trait association was declared significant when the mean or median values of the two marker classes were significantly different at $P<0.05$ in two locations or two independent tests. Otherwise $P<0.01$ was selected as threshold.

3. RESULTS

3.1 Mapping functional genes involved in carbohydrate metabolism and transport

3.1.1 PCR amplification of functional genes through an STS approach

Genes involved in starch and sucrose metabolism, metabolite transport, Calvin cycle, oxidative pentose phosphate pathway, glycolysis and TCA cycles were explored in public databases of EMBL and GenBank for their sequences in potato, tomato or other species. 51 gene-specific primer pairs were designed (Table 3-1, page 51-55), of which 45 primer pairs were derived directly from the DNA sequence of potato genes and three pairs from tomato genes (*Eno*, *Ndpk*, and *Glo*). For genes *Ant*, *Rca and* Pgk_{cp}, which were not available either as a potato or as a tomato sequence, degenerate primers were designed based on sequence motifs conserved between the homologous genes of three to five other plant species (Schneider *et al*. 1999, Van Campenhout *et al*. 2000). The consensus sequence of a gene can be revealed by sequence analysis using the *Pile-Up* program of *GCG* software package (University of Wisconsin Genetics Computer Group, version 8). The example of a multiple sequence pileup of sequences of the *Pgk* gene from several species is shown in Figure 3-1 (on page 47-49). Using potato genomic DNA as a template, most primer pairs generated a single fragment, only a few produced two fragments. Most frequently the PCR products were larger than expected from the transcript sequence, indicating the presence of introns.

```
        551                                                   600
u37701  CGAAAGCTGA TGATTGTATT GGCCCAGAAG TGGAAAGCTT GGTGGCTTCT
z48977  TGAAGGTTGA GGACTGCATT GGTCCAGAAG TTGAGAAGTT GGTTGCTTCA
x68430  TGAAGGCTGA TGATTGCATT GGTCCAGATG TTGAGAAGTT GGTTGCTGAA
x15233  AAAAAGCAGA AGATGTTATC GGCCCAGAAG TTGAGAAATT GGTGGCTGAC
          ** *  **  **    **  ** ***** * * ** *  ** *** ***
        601                                                   650
u37701  CTACCTGAAG GTGGAGTTTT GCTTCTTGAG AACGTCAGGT TTTACAAGGA
z48977  CTTCCCGAGG GTGGTGTTCT TCTTCTCGAG AACGTGAGAT TCTACAAGGA
x68430  CTCCCAGAAG GTGGCGTTCT TCTCCTTGAG AATGTTAGGT TCTACAAGGA
x15233  CTGGCAAATG GTGCTGTTTT GCTCCTGGAA AACGTAAGAT TTTACAAGGA
        **  *  * * ***  *** *  ** ** **  ** ** ** * * ********
        651                                                   700
u37701  GGAAGAGAAG AACGATCCTG AGTTTGCTAA GAAGCTTGCT TCTCTAGCTG
z48977  GGAAGAGAAG AACGAACCTG AGTTTGCAAA GAAACTTGCA TCATTGGCAG
x68430  AGAGGAAAAG AATGATCCAG AATTTGCAAA GAAGCTTGCT TCCTTGGCAG
x15233  AGAGGAGAAG AATGACCCAG AGTTTGCAAA GAAGCTTGCG TCACTGGCAG
         ** ** *** ** ** ** * * ***** ** *** *****  **  * ** *
```

```
        701                                                       750
u37701  ACCTTTATGT CAATGATGCT TTCGGAACTG CTCACAGAGC TCATGCTTCT
z48977  ATCTTTATGT GAATGATGCA TTCGGTACAG CTCACAGAGC ACATGCCTCT
x68430  ATCTGTATGT GAATGATGCT TTTGGCACTG CCCACAGAGC TCATGCATCA
x15233  ATCTGTTTGT AAACGATGCA TTCGGAACAG CCCACAGAGC ACATGCCTCG
        * ** * ***  ** *****  ** ** ** * * ********  ***** **
        751                                                       800
u37701  ACCGAAGGAG TCACTAAGTT CTTGAAGCCT TCAGTTGCTG GTTTCCTTTT
z48977  ACAGAGGGAG TTACTAAATT TTTGAAGCCT TCTGTTGCAG GTTTCCTCTT
x68430  ACCGAGGGAG TTACCAAATT TTTGAAGCCA TCTGTTGCTG GCTTCCTCTT
x15233  ACTGAGGGAG TTACCAAGTT CTTGAAGCCT TCTGTTGCTG GATTCCTTTT
        ** ** **** * ** ** **  ********  ** ***** * * ***** **
        801                                                       850
u37701  GCAAAAGGAA CTGGACTACC TAGTTGGTGC TGTTTCAAAC CCAAAGAGAC
z48977  ACAAAAGGAA TTGGACTATT TAGTCGGGGC GGTTTCAAAT CCAAAGAGGC
x68430  ACAGAAGGAA TTGGACTACC TTGTTGGAGC TGTTTCTAAC CCAAAGAGAC
x15233  GCAGAAGGAA CTTGACTACC TGGATGGAGC TGTTTCA AAC CCTAAGCGCC
         ** ******  * *****   * *  ** **  ***** AAY CCWAAGMGVC
                                                  degenerate primer
        851                                                       900
u37701  CATTTGCAGC CATAGTGGGT GGTTCCAAAG TCTCATCCAA GATTGGAGTT
z48977  CATTTGCTGC TATTGTGGGT GGTTCAAAGG TTTCATCCAA GATTGGAGTG
x68430  CATTTGCTGC TATAGTTGGT GGTTCAAAGG TCTCTTCCAA AATTGGAGTA
x15233  CATTTGCTGC CATTGTGGGT GGCTCAAAGG TGTCATCCAA GATTGGGGTT
        CATTTGC **  ** ** *** ** ** ** * * ** ***** ****** **
        901                                                       950
u37701  ATTGAATCGC TTCTGGAGAA GTGTGATATT CTTCTTCTTG GTGGTGGAAT
z48977  ATCGAATCAC TTTTAGAGAA ATGTGATATA TTGCTTTTGG GTGGAGGAAT
x68430  ATTGAGTCAC TTTTGGAGAA ATGTGATATC CTACTCCTTG GAGGAGGAAT
x15233  ATCGAATCCC TGTTGGAGAA GTGTGACATC CTTCTTTTGG GTGGTGGTAT
        ** ** ** * *  * *****  ***** **   * **  * * * ** ** **
        951                                                      1000
u37701  GATCTTCACA TTCTACAAGG CACAGGGTCT TTCAGTTGGT TCGTCCCTTG
z48977  GATCTTTACC TTCTACAAGG CTCAGGGTCT TTCAGTTGGT TCCTCCTTGG
x68430  GATCTTTACA TTCTACAAAG CTCAAGGTAT GTCAGTTGGA TCTTCTCTGG
x15233  GATCTTCACA TTTTACAAGG CACAAGGACT CTCAGTTGGT TCTTCCTTGG
        ****** **  ** ***** * * ** **  *  ********  ** **  * *
        1001                                                     1050
u37701  TTGAAGAAGA CAAGCTTGAA TTGGCTACAG AACTCCTTGC CAAAGCTAAG
z48977  TTGAGGAAGA CAAACTAGAA CTCGCTACAT CACTCCTAGA GAAGGCCAAG
x68430  TTGAAGAGGA TAAGCTTGAC CTTGCAACTT CTCTCCTCGC CAAGGCTAAG
x15233  TTGAGGAAGA TAAACTTGAG CTGGCAACAT CTCTCCTTGC AAAGGCAAAG
        **** ** **  ** ** *    * ** **     *****     **  * ***
        1051                                                     1100
u37701  GCCAAAGGAG TCTCTCTTTT GTTGCCAACA GATGTTGTAG TTGCTGACAA
z48977  GCGAAAGGAG TCAGTCTCTT GTTACCATCT GATGTTGTGA TTGCAGATAA
x68430  GAAAAGGGCG TGTCTCTGTT GTTGCCTACT GATGTCGTTA TTGCGGACAA
x15233  GCAAAGGGTG TCTCCCTTCT GTTGCCATCT GACGTTATCA TTGCTGATAA
        *  ** ** * *    **  * *** **  *  ** **  *   **** ** **
        1101                                                     1150
u37701  GTTTGCTCCT GATGCCAACA GCAAGATTGT GCCTGCATCA GGCATTGAGG
z48977  ATTTGCTCCT GATGCAAACA GCAAGATTGT GCCTGCATCT GCTATCCCAG
x68430  GTTTGCTGCT GATGCAGACA GCAAGATTGT CCCCGCATCT GGAATCCCTG
x15233  GTTTGCTCCT GATGCTAACA GTCAGACCGT CCCTGCATCT GCAATTCCTG
         ****** ** *****  *** *  ***  **  ** *****  *  **    *
        1151                                                     1200
u37701  ACGGATGGAT GGGACTGGAC ATTGGTCCAG ACTCTATCAA AACTTTCAAC
z48977  ACGGTTGGAT GGGGTTGGAC ATTGGACCAG ACTCTGTTAA GACTTTCAAC
x68430  ACGGATGGAT GGGATTGGAC ATTGGACCTG ATTCTATTAA GACATTTAGT
x15233  ATGGTTGGAT GGGGCTGGAC ATTGGCCCAG ATTCAGTCAA AACATTTAAT
        * **  **** ***  ***** ***** ** * * **  * **  ** **
```

```
        1201                                                    1250
u37701  GAAGCTCTGG ACACAACACA AACAGTCATT TGGAATGGAC CTATGGGAGT
z48977  GATGCCTTGG ATACCACAAA AACAGTGATC TGGAATGGAC CTATGGGGGT
x68430  GAAGCTTTGG ACACTACCCA GACCGTCATC TGGAATGGTC CCATGGGAGT
x15233  GATGCCCTGG ACACAACACA GACAATCATT TGGAACGGAC CAATGGGTGT
        ** **  *** * ** **  *  **  * **  ***** ** * * ***** **
        1251                                                    1300
u37701  TTTCGAGATG GAAAAGTTTG CGGCTGGAAC AGAGGCGATA GCGAATAAAC
z48977  GTTTGAATTT GACAAGTTTG CTGTTGGAAC AGAGGCAATT GCAAAGAAGC
x68430  TTTTGAATTC GAAAAGTTTG CTGCTGGTAC AGAGGCTATT GCTAAGAAGC
x15233  CTTTGAATTT GACAAGTTTG CAGTAGGAAC TGAGTCTATT GCAAAGAAGT
         **  *  *  ** ******* * *  ** **  *** * **  ** ** **
        1301                                                    1350
u37701  TAGCAGAGCT AAGTGAAAAA GGAGTGACAA CGATAATAGG AGGAGGAGAC
z48977  TCGCGGACTT AAGTGGGAAA GGAGTGACGA CTATCATTGG AGGTGGAGAT
x68430  TAGAGGAGAT AAGCAAGAAG GGTGCAACCA CAATTATCGG TGGTGGTGAC
x15233  TGGCCGAGCT TAGCAAAAAG GGTGTGACAA CTATCATTGG AGGCGGAGAC
        *     *  *   *    **  ** *  ** * * ** ** **  ** ** **
        1351                                                    1400
u37701  TCAGTGGCTG CAGTGGAGAA AGTAGGAGTA GCAGGAGTCA TGAGTCACAT
z48977  TCTGTTGCAG CTGTTGAGAA AGTTGGAGTT GCTAGCGTGA TGAGCCACAT
x68430  TCAGTTGCAG CAGTAGAGAA GGTAGGAGTG GCAGAGGCAA TGAGCCACAT
x15233  TCCGTTGCGG CTGTTGAGAA GGTGGGAGTG GCTGATGTTA TGAGCCACAT
        ** ** ** * * ** *****  ** *****  **    *  * ****  ****
        1401                                                    1450
u37701  CTCCACTGGT GGTGGAGCCA GCTTGGAGCT GTTGGAAGGA AAAGTACTTC
z48977  ATCCACTGGT GGTGGTGCCA GTTTGGAGCT ACTGGAAGGC AAGGTGCTCC
x68430  ATCAACTGGT GGTGGAGCTA GTTTGGAGTT GTTAGAAGGG AAACAGCTTC
x15233  CTCAACTGGT GGTGGCGCCA GCTTGGAGTT GTTGGAAGGA AAGGAGCTTC
         ** TGACCA CCACCDCGRT CRAA**** *   * *****  **    ** *
            degenerate primer
        1451                                                    1500
u37701  CCGGTGTGAT CGCCCTTGAT GAAGCAATCC CAGTCACTGT TTAGAAACTC
z48977  CCGGTGTCAT TGCTCTTGAT GAAGCAGACG CCCCCGTTGC TGTGTAAAAC
x68430  CTGGAGTACT TGCTCTTAAT GAGGCCGACC CAGTTCCGGT TTAAGACCAC
x15233  CTGGAGTCGT TGCACTTGAT GAAG..GTGT CATGACGAGG TCGGTGACCG
        * ** **  *  ** *** ** ** *       *          *
        1501                                                    1550
u37701  TTACCATCTA TAAGGCAAAC GCTGTATACT TAAAAGAGTT TCTTTCGCTT
z48977  AATTTGTACT AATTCTTTTT TCTCCGGGGT CAACATAATC AGTGGTAATT
x68430  TCTATACAAA TTCTTTTTTT CCCCCTCTCT TCACAGGTTC ATCCTCTTAA
x15233  TATGAGGCTA AGCTTCATTT GTTGCATCTT AATTCCTTTC ATGTACCGTT
                                        *         *
```

Figure 3-1 Multiple alignment of partial cDNA sequences of the gene coding for phosphoglycerate kinase (PGK) from 4 different species showing the conserved sequence motifs used as primers for amplifying homologous genes in potato. U37701, Z48977, X68430 and X15233 are cDNA sequences from *Arabidopsis*, tobacco, spinach and wheat, respectively. Asterisks indicate identical positions. Sequence motifs adopted for primers are highlighted in bold.

3.1.2 Polymorphism Identification

Most primer pairs were selected to flank introns which are potentially more variable when compared to exons (Tragoorung *et al.* 1992; Konieczny and Ausubel 1993; Corte-Real *et al.* 1994). This allowed efficient identification of polymorphisms by SCAR or CAPS. In total, four primer pairs for genes *SssI*, *Pk*, *Me* and *Ndpk* generated directly polymorphic PCR

products of which *Me* detected two loci (SCAR markers, Table 3-1, page 51-55). PCR products of 45 genes were digested with a 4-base pair cutter restriction enzyme to generate segregating DNA fragments (CAPS markers, Figure 3-2). 43 of the 45 genes were mapped as CAPS markers using one of the following enzymes *Taq*I, *Rsa*I and *Alu*I. The remaining two genes were mapped using *Hae*III and *Dde*I. The most informative restriction enzyme was *Taq*I followed by *Rsa*I and *Alu*I (Table 3-1, page 51-55). PCR products of all the genes were restricted with at least these three enzymes for CAPS to maximize the identification of loci even for the ones showing directly SCAR polymorphism. However, the increase of polymorphic fragments produced by different restriction enzymes did not increase the number of loci identified. Frequently, these polymorphic fragments, which were derived from the same genes, tagged an alternative allele of one parent or the other or common alleles of both. In the case of *Pain*-1 and *Eno* for example, CAPS assays with the enzymes *Dde*I and *Taq*I for *Pain*-1 and *Alu*I and *Taq*I for *Eno* resulted in 3 polymorphic fragments with different segregation patterns which were all mapped at a single locus on linkage groups (LG) III and IX, respectively. The only exceptions was *Ppe* which identified three loci, two (LG III, LG V) by *Alu*I and one (LG XII) by *Taq*I. Other genes with two loci being identified are *Pha*1, *Glo* and *Fum*1 by *Taq*I and *Rsa*I (Table 3-1, on page 51-55).

The PCR product of only one gene (*Tk*), which may contain no introns in the region investigated, did not segregate either as SCAR or as CAPS marker. Alternatively, by RFLP analysis using the PCR product as the probe, the *Tk* gene was successfully mapped on LG V (Table 3-1, on page 51-55). ***(continue to page 56)***

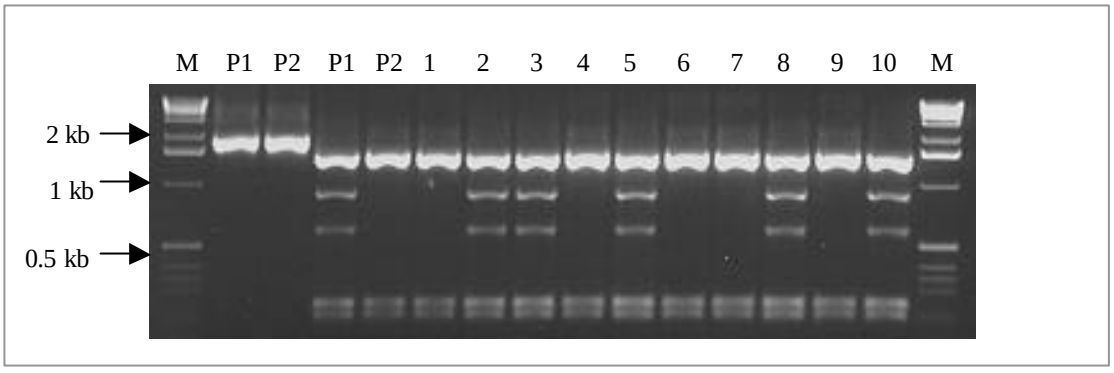


Figure 3-2 CAPS marker for the a**-glucan phosphorylase gene *Stp*23 which was mapped on potato chromosome III.** The uniform 2 kb PCR products, shown for the parents P1 and P2 of the LH mapping population (2nd and 3rd lane from left), were polymorphic after digestion with *Taq*I (4th and 5th lane) and segregated in the F1 plants (6th to 15th lane from left). M = size marker.

Table 3-1 Genes placed onto the potato molecular function map

Enzyme	EC number	Accession number	Reference	Gene	Linkage group	Primer, 5' to 3'	Popu-lation[a]	PCR product kb	Marker Assay[b]
A: Starch synthesis and degradation (chloroplasts, amyloplasts)									
ADP-glucose pyrophosphorylase B[e]	2.7.7.27	X55155	Mueller-Rober *et al*. 1990	*AGPaseB*	VII, XII	-	1,2,3,4	-	R, TaqI
ADP-glucose pyrophosphorylase S[e]	2.7.7.27	X61186	Nakata *et al*. 1991	*AGPaseS*	I, IV, VIII	-	1,2,3,4	-	R, RsaI, TaqI
Granule bound starch synthase I[e]	2.4.1.21	X52416	Rohde *et al*. 1990	*GbssI (wx)*	VIII	-	1, 3, 4	-	R, RsaI
Soluble starch synthase I	2.4.1.21	Y10416	Abel *et al*. unpublished	*SssI*	III	f-cttcttactgcagacctggaac r-ctgtcagtatccgatcagcaac	3	2.4 2.6	S
Granule bound starch synthase II	2.4.1.21	X87988	Edwards *et al*. 1995	*GbssII*	II	f-gctgcaagtgctgatgaatcga r-ttagaccatggagcgcattctg	3	1.8	C, TaqI
Soluble starch synthase III	2.4.1.21	X94400	Abel *et al*. 1996	*SssIII*	II	f-aacaaaagttcaggtcctctctc r-aaatcccaccatcttctctctc	3	1.3	C, AluI
Starch branching enzyme I[e]	2.4.1.18	Y08786	Khoshnoodi *et al*. 1996	*SbeI*	IV	-	1, 3, 4	-	R, TaqI
Starch branching enzyme II	2.4.1.18	AJ000004	Larsson *et al*. 1998	*SbeII*	IX	f-ctcgtagtgctacaggtatcac r-tgatggagtgtccatacgta	3	1.8	C, AluI
Disproportionating enzyme	2.4.1.25	X68664	Takaha *et al*. 1993	*Dpe-P*	IV	f-cactacttttcaatctcctatccc r-gcatagtcacgaacttttttcc	3	3.0	C, TaqI
Starch-granule-bound protein R1	-	Y09533	Lorberth unpublished	*Gb-R1*	V	f-tccatcctgagactggagatac r-acttgtactgcaggactggaag	4	1.7	C, RsaI
Debranching enzyme	3.2.1.41	A52190	Kossmann patent	*Dbe*	XI	f-ctgatgtcagcatctatgagct r-gatacgacaaggaccatttgca	3 4	2.1	C, TaqI R, AluI
α-Amylase[e, g]	3.2.1.1	-		*AmyZ*	IV	-	1, 2	-	R, TaqI
α-Glucosidase	3.2.1.20	AJ001374	Taylor *et al*. 1998	*Agl*	IV	f-accaagctgtggttaaccagag r-gcagttgcgaataactgtggca	3	0.8	C, AluI
H-type starch phosphorylase	2.4.1.1	-[f]		*StpH*	IX	f-gcatactatgctgctactgctg r-gcacatcatatgcaagagcctg	3	1.8	C, RsaI
L-type starch phosphorylase	2.4.1.1	X73684	Sonnewald *et al*. 1995	*Stp1*	V	f-acacactatgttctgcttctcttc r-actatcctccacctcaaccttc	4	0.8	C, TaqI RsaI

Table 3-1 Genes placed onto the potato molecular function map (continued)

Enzyme	EC number	Accession number	Reference	Gene	Linkage group	Primer, 5' to 3'	Popu-lation [a]	PCR product kb	Marker Assay [b]
α-Glucan phosphorylase	2.4.1.1	D00520	Nakano *et al*. 1989	*Stp23*	III	f-atggcgactgcaaatggagca r-ccatacttgtacctaagtccatag	4	1.6	C, TaqI, RsaI, AluI
B: Sucrose metabolism (cytosol, apoplast)									
UDP-glucose pyrophosphorylase [c]	2.7.7.09	U20345	Borovkov unpublished	*UGPase*	XI	-	1, 2, 4	-	R, TaqI
Fructose-1,6-bisphosphatase	3.1.3.11	X76946	Zrenner *et al*. 1996	Fbp_{cy}	IV	f-tgcagggagaagatcaaaagaaac r-tgaagaaccatcagcgggatac	3	1.4	C, RsaI TaqI
Pyrophosphate fructose-6-phosphate 1-phosphotransferase, α subunit	2.7.1.90	M55190	Carlisle *et al*. 1990	*Pfp-a*	IV	f-cggacaaaagatcagattagaacgac r-ccctcaggcaacaggataacac	3	2.5	C, AluI
Pyrophosphate fructose-6-phosphate 1-phosphotransferase, β subunit	2.7.1.90	M55191	Carlisle *et al*. 1990	*Pfp-b*	II	f-gtcatgatagatgctcgatcaac r-acatcagcaatgtagtccgtaac	4	2.5	C, RsaI
Sucrose phosphate synthase [g]	2.4.1.14	X73477	Zrenner *et al*. 1995	*Sps*	VII	f-gaaagaggtcgcagagaagcag r-caagtgagtgaccagtgaaaag	2, 4	1.3	C, TaqI R, TaqI
Sucrose synthase 3	2.4.1.13	U24088	Fu *et al*. 1995	*Sus3*	VII	f-catgacaaggaaagcatgacccc r-gcaaagtaaatcttatacatghtgacc	4	1.2	C, TaqI
Sucrose synthase 4 [k]	2.4.1.13	U24087	Fu *et al*. 1995	*Sus4*	XII	f-caagctgacctggacaccacagt r-accacattgaaaaccataggaattct	3	1.3	C, RsaI
Soluble acid invertase	3.2.1.26	X70368	Zrenner *et al*. 1996	*Pain-1*	III	f-gtcttcgataagacttttcgag r-cagtggtcgggtctctaaagt	3	2.0	C, DdeI TaqI
Apoplastic invertase	3.2.1.26	Z22645	Hedley et al. 1994	Inv_{ap}	IX, X	-	1	-	R, TaqI
C: Transport (membranes)									
Sucrose transporter	-	X69165	Riesmeier *et al*. 1993	*Sut1*	XI	f-agcttccatagctgctggtgtt r-cggactaaggttaaggctatgg	1, 3	1.2	C, RsaI R, TaqI
Triose phosphate translocator [e]	-	-		*Tpt*	X	-	2	-	R, RsaI
Glucose-6-phosphate translocator	-	AF020816	Kammerer *et al*. 1998	*Gpt*	V	f-ggctcacacaattggtcatgtg r-ccaagattgcaatagcagcacc	4	1.4	C, HaeIII
Plasma membrane H^+-ATPase 1 [e]	3.6.1.34	X76536	Harms *et al*. 1994	*Pha1*	III, VI	f-tcctggagatggtgtctactct r-gcagtatcaatggcatcctggt	2, 3	0.8	C, TaqI R, TaqI

Table 3-1 Genes placed onto the potato molecular function map (continued)

Enzyme	EC number	Accession number	Reference	Gene	Linkage group	Primer, 5' to 3'	Popu-lation [a]	PCR product kb	Marker Assay [b]
Plasma membrane H^+-ATPase 2 [e]	3.6.1.34	X76535	Harms *et al*. 1994	*Pha2*	VII	f-caaacatgtacccgtcagcatc r-agctatcaggcattggagatgg	1, 4	0.9	R, TaqI C, TaqI
Adenylate transporter	-	X65549 X62123 D12637 X95864 X68592		*Ant*	XI	f-tggagaggaaacactgcYaatgt [l] r-atgttRgcaccagcWcccttga [l]	4	1.1 + 0.9	C, TaqI
Inorganic phosphate transporter 1	-	X98890	Leggewie *et al*. 1997	*Pt1*	IX	f-tcagcagctgttaatggagtcg r-tgcaacaccttggacatgtcgt	4	0.6	C, RsaI
Inorganic phosphate transporter 2	-	X98891		*Pt2*	III	f-cgccacgatcatgtctgaatac r-taaacgccacggtgaaccagta	4	0.7	C, AluI
D: Calvin cycle, photorespiration (chloroplasts)									
Ribulose bisphosphate carboxylase activase	-	J03610 AF037361 X14212 Z21794		*Rca*	X	f-acacYgtMaacaaccagatg [l] r-actctcttgacattctcttgc	4	0.7	C, TaqI
Ribulose bisphosphate carboxylase small subunit [e]	4.1.1.39	- [i]		*rbcS*	II (2) III	-	1,3,4	-	R, TaqI, RsaI
Phosphoglycerate kinase	2.7.2.3	U37701 Z48977 X68430 X15233		Pgk_{cp}	VII	f-aaYccWaagMgVccatttgc [l] r-aaRctRgcDccaccaccagt [l]	4	0.9	C, TaqI
Fructose-1,6-bisphosphatase	3.1.3.11	- [h]	Kossmann *et al.* 1992	Fbp_{cp}	IX	f-tactggagttcaaggtgctgtc r-tgcagagtaaggcttccgacta	3	1.6	C, TaqI
Transaldolase	2.2.1.2	U95923	Moehs *et al*. 1996	*Tal1*	XI	f-attccttgtgtgtcaaatgctcc r-cgactaacgaagaatgaagcaac	4	1.5	C, TaqI
Transketolase	2.2.1.1	Z50099	Teige *et al*. 1995	*Tk*	V	f-gagacacggaaattgctttcac r-tcagcactaccaccaaggaaac	4	0.6	R, TaqI
Pentose-5-phosphate 3-epimerase	5.1.3.1	Z50098	Teige *et al*. 1995	*Ppe*	III, V, XII	f-tccgtccatcctttctgctaac r-aactccaccatcaacttcaatc	3	3.0	C, AluI TaqI
Glycolate oxidase	1.1.3.15	X92888	Speirs *et al*. 1995	*Glo*	VII, X	f-cagcgttgaggaggttgcttca r-gacagccactcaatgccatagt	4	1.7	C, TaqI

Table 3-1 Genes placed onto the potato molecular function map (continued)

Enzyme	EC number	Accession number	Reference	Gene	Linkage group	Primer, 5' to 3'	Popu-lation [a]	PCR product kb	Marker Assay [b]
E: Glycolysis, oxidative pentose phosphate pathway (cytosol)									
Fructokinase	2.7.1.4	Z12823	Smith *et al*. 1993	*Fk*	VI	f-gctttggcgttcgtgactctac r-agtggtgtcaacagtcttcacg	3	2.0	C, TaqI
Hexokinase [g]	2.7.1.1	X94302	Clericus unpublished	*Hxk*	III	f-gattatattgcggctgagcttgc r-catctgccgttgacagagtatg	2, 3	0.8	C, RsaI R, TaqI
Glyceraldehyde 3-phosphate dehydrogenase [d]	1.2.1.12	U17005	Schneider *et al*. 1995	*Gap C*	V	-	1	-	R, TaqI
Enolase	4.2.1.11	X58108	Van der Straeten *et al*. 1991	*Eno*	IX	f-cttggtgcaaatgccatccttg r-cagcttcaatactctcggtcac	3	2.1 + 1.9	C, AluI TaqI
Pyruvate kinase	2.7.1.40	X53688	Blakeley *et al*. 1990	*Pk*	IV	f-tcactgtatccacagactatacc r-cactctccccacttaacataac	3	0.9 0.8	S
Lactate dehydrogenase 2 [g]	1.1.1.28	AF067859	Dunford unpublished	*Ldh2*	VIII (3)	-	2	-	R, TaqI
Phosphoenolpyruvate carboxylase	4.1.1.31	X67053	Merkelbach *et al*. 1993	*Ppc*	X, XII	-	1	-	R, TaqI
Glucose-6-phosphate dehydrogenase	1.1.1.49	X74421	Graeye *et al*. 1994	*G6pdh*	II	f-tcttctattttgctcttcctcc r-actcgtatttctgcctttcttg	3	1.6	C, DdeI
F: TCA cycle (mitochondria)									
Pyruvate dehydrogenase, E1α	1.2.4.1	Z26949	Grof *et al*. 1995	Pdh-E1α	V	f-tcaacaagccgagccattaac r-gattagcagcaccatcaccatac	3	0.6	C, RsaI
Citrate synthase	4.1.3.7	X75082	Landschutze *et al*. 1995	*Cis*	I	f-ggtcacttggttgctagtgctt r-ctttcgcagaactccatgtcca	3	1.5	C, TaqI
Aconitase	4.2.1.3	X97012	Surpili unpublished	*Aco*	VII	f-catggctcctgaatatggtgca r-atacaagtggtgcagccatagc	4	1.3	C, AluI HpaII
Isocitrate dehydrogenase 1	1.1.1.42	X75638	Fieuw unpublished	*Icdh-1*	I	f-aaaatatccccagacttgtccc r-aacagcgtcaatgaactcttcc	3	1.8	C, AluI
Fumarase	4.2.1.2	X91615	Nast unpublished	*Fum1*	VII, IX	f-catgtgcatccaaatgaccatgt r-gaagcagccagggtatttaagg	4	1.9	C, RsaI
Malate dehydrogenase	1.1.1.39	Z23023	Winning *et al*. 1994	*Mdh*	VIII	f-acagtggattgttcagacgtcc r-gtcaagccgatgatcttgcagt	4	3.0	C, AluI

Table 3-1 Genes placed onto the potato molecular function map (continued)

Enzyme	EC number	Accession number		Gene	Linkage group	Primer, 5' to 3'	Popu-lation[a]	PCR product kb	Marker Assay[b]
NAD^+-dependent malic enzyme	1.1.1.39	Z23002	Winning *et al*. 1994	*Me*	III, V	f-ccttggttcaataaggacacgg r-caacgagcatgaacagcttcca	4	1.1 1.2	S
G: Miscellaneous									
Soluble inorganic pyrophosphatase[e]	3.6.1.1	Z36894	Du Jardin *et al*. 1995	*Ppa1*	VIII, XII	-	1	-	R
GDP-mannose pyrophosphorylase	2.7.7.13	AF022716	Keller unpublished	*GMPase*	III	f-gaaactgaaccacttggcactg r-ccctctccaatcttggcagatt	4	0.6	C, AluI
Uncoupling protein	-	Y11220	Laloi *et al*. 1997	*Ucp*	IX	f-gtatcgtacctgggttacatcg r-acattccaagatcccaagcgtc	3	2.5	C, TaqI
Pectin methyl esterase	3.1.1.11	S66607	Pear *et al*. 1993	*Pme*	VII	f-acttgctgaagcagttgctgca r-ttagccacagtgaacggcatag	4	1.5, 1.2, 1.0	C, TaqI
3-deoxy-D-arabino-heptulosonate 7-phosphate synthase	4.1.2.15	M95201	Zhao *et al*. 1993	*ShkB*	XI	f-catcttctccataacccttacc r-gctcacagttctccacaaaatc	3	1.2	C, RsaI
Nucleoside diphosphate kinase	2.7.4.6	X75324	Harris *et al*. 1994	*Ndpk*	IX	f-atgatcaagcctgatggtgtcca r-aagagtgaaggctgctctgcca	3	1.1 0.4	S
β-1,3-glucanase, basic[e]	3.2.1.39	AJ009932	Talarczyk *et al*. 1998	*Glu B*	I	f-cagcctatcggagtatgctatg r-cattgaatccacaagggcatcg	3 1	0.7	C, RsaI R
β-1,3-glucanase, acidic	3.2.1.39	-		*Glu A*	I	-	1	-	R, TaqI
Chitinase, class I, basic[e]	3.2.1.14	-		*Cht B*	X	-	1, 3, 4	-	R, TaqI
Protein kinase	2.7.1.37	X90990	Meyer *et al*. 1996	*Prk*	II	f-ccatctgagtcaggaatcacct r-acattgatcgagcactcgttgg	3	1.6	C, RsaI

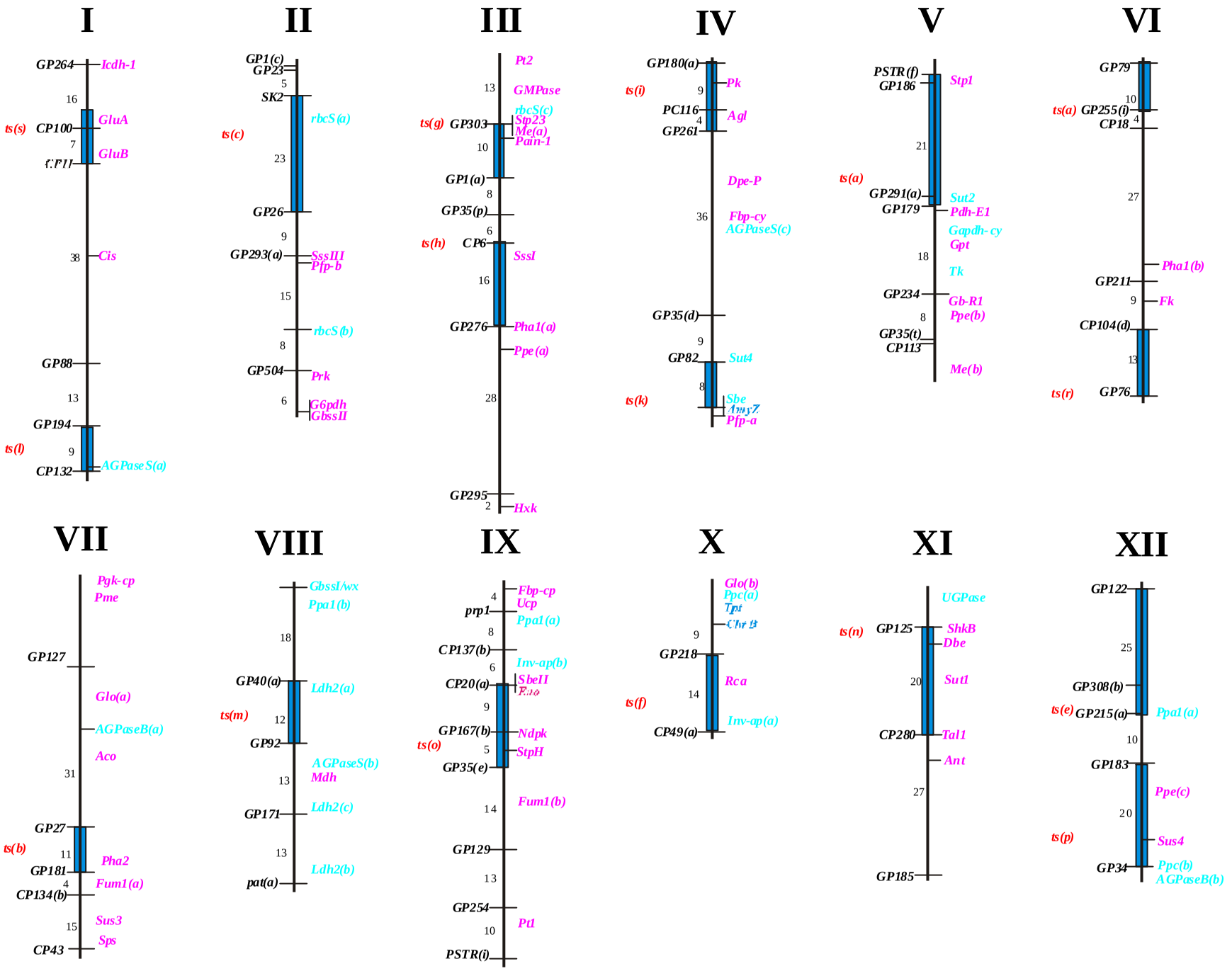
[a] Four populations were used for mapping: 1 = BC916[2], 2 = F1840 (Gebhardt *et al.* 1991, 1994), 3 = K31, 4 = LH (Schäfer-Pregl *et al.* 1998)
[b] C = CAPS, S = SCAR, R = RFLP
[c] The sequence of RFLP marker *CP58* originating from leaf cDNA (Gebhardt *et al.* 1991) codes for the 3' end of potato UGPase.
[d] The sequence of the cold regulated cDNA CI13 isolated from cold stored tubers (van Berkel *et al.* 1994) codes for GAP C (Schneider 1995).
[e] Map position of this gene has been reported previously (Gebhardt *et al.* 1994, Du Jardin *et al.* 1995), they have been included in the Table based on their role in carbohydrate metabolism or transport.
[f] Sequence from Mori *et al.* 1991
[g] Potato EST clone (unpublished results from this laboratory) was used as probe for RFLP mapping
[h] Sequence from Kossmann *et al.* 1992
[i] Sequence from Eckes *et al.* 1985, and Wolter *et al.* 1988
[k] The *Sus4* gene of potato corresponds to *sh1* of maize (Gebhardt *et al.* 1991, Werr *et al.* 1985)
[l] Y = C + T, W = A + T, M = A + C, R = A + G, V = A + C + G, D = A + G + T

(continued from page 50)

3.1.3 Segregation analysis and function map construction

To localise the genes, segregation data were created by scoring 1 for the presence and 0 for the absence of DNA fragments. When multiple polymorphic fragments were detected for a single gene, they were scored separately and all were subjected to linkage analysis without considering their possible allelism. The software program MAPRF© (E. Ritter, Version 2.1) was employed for segregation and linkage analysis in conjunction with previously generated RFLP marker framework data sets of the same mapping populations K31 or LH. Depending on the parental genotypes, two segregation patterns were expected for any given polymorphic fragment. One is a 1:1 ratio when the fragment is present in only one of the parents in heterozygous state, the other is a 3:1 ratio (presence versus absence) when the fragment originated from both parents. For the map construction, each gene was initially assigned to either a male or female linkage group depending on the donor parent of the fragments. A composite linkage map was subsequently constructed by integrating the male and female linkage groups based on common markers. The average recombination values between markers of the two parental maps were converted into map distance centiMorgans (cM) using Kosambi's mapping function (Kosambi 1944). The integrated function map of K31 is adopted to be the final function map shown in Figure 3-3 (on page 57). The genes mapped in the LH population are shown at their approximate position based on anchor RFLP markers between the two maps.

Figure 3-3 Molecular function map of potato. The twelve potato chromosomes are shown as a composite map based on the K31 mapping population. Genetic distances, indicated in centiMorgans on the left side of the linkage groups, are the means of the same marker intervals on maternal and paternal linkage groups (Schäfer-Pregl *et al*. 1998). Framework RFLP markers (Gebhardt *et al*. 1994) are positioned to the left side of the linkage groups and gene markers are positioned on the right side (for gene identification see Table 3-1). Relative positions of genes mapped in populations other than K31 (not connected to the vertical linkage group with a horizontal bar, see Table 3-1 for details) were inferred from reference RFLP markers. Small letters in parentheses indicate that more than one locus was identified with the same probe or PCR primer pair. Genes mapped by RFLP and STS approaches are shown in light blue and pink respectively. Map segments having QTL for TSC are shown as blue bars. QTL positions *ts(a)* to *ts(s)*, as identified by interval mapping (Schäfer-Pregl *et al*. 1998) are shown on the left side of the linkage groups.


I
GP264
Icdh-1
16
ts(s)
CP100
GluA
7
GluB
38
Cis
GP88
13
GP194
ts(l)
9
CP132
AGPaseS(a)
II
GP1(c)
GP23
5
SK2
rbcS(a)
ts(c)
23
GP26
9
GP293(a)
SssIII
Pfp-b
15
rbcS(b)
8
GP504
Prk
6
G6pdh
GbssII
III
Pt2
13
GMPase
rbcS(c)
ts(g)
GP303
Stp23
Me(a)
Pain-1
10
GP1(a)
8
GP35(p)
6
ts(h)
CP6
SssI
16
GP276
Pha1(a)
Ppe(a)
28
GP295
2
Hxk
IV
GP180(a)
ts(i)
9
Pk
PC116
Agl
4
GP261
Dpe-P
36
Fbp-cy
AGPaseS(c)
GP35(d)
9
GP82
Sut4
8
ts(k)
Sbe
AmyZ
Pfp-a
V
PSTR(f)
GP186
Stp1
21
ts(a)
GP291(a)
Sut2
GP179
Pdh-E1
Gapdh-cy
Gpt
18
Tk
GP234
Gb-R1
Ppe(b)
8
GP35(t)
CP113
Me(b)
VI
GP79
10
ts(a)
GP255(i)
CP18
4
27
Pha1(b)
GP211
9
Fk
CP104(d)
13
ts(r)
GP76
VII
Pgk-cp
Pme
GP127
Glo(a)
AGPaseB(a)
Aco
31
GP27
ts(b)
11
Pha2
GP181
4
Fum1(a)
CP134(b)
15
Sus3
Sps
CP43
VIII
GbssI/wx
Ppa1(b)
18
GP40(a)
Ldh2(a)
ts(m)
12
GP92
AGPaseS(b)
Mdh
13
GP171
Ldh2(c)
13
pat(a)
Ldh2(b)
IX
4
Fbp-cp
Ucp
prp1
Ppa1(a)
8
CP137(b)
6
Inv-ap(b)
CP20(a)
SbeII
Ent
9
GP167(b)
Ndpk
ts(o)
5
StpH
GP35(e)
Fum1(b)
14
GP129
13
GP254
Pt1
10
PSTR(i)
X
Glo(b)
Ppc(a)
Tpt
Chr1
9
GP218
Rca
14
ts(f)
CP49(a)
Inv-ap(a)
XI
UGPase
ts(n)
GP125
ShkB
Dbe
20
Sut1
CP280
Tal1
Ant
27
GP185
XII
GP122
25
GP308(b)
ts(e)
GP215(a)
Ppa1(a)
10
GP183
Ppe(c)
20
ts(p)
Sus4
GP34
Ppc(b)
AGPaseB(b)

3.1.4 Homology of PCR products to the reference sequences

To verify the similarity of PCR products to the genes investigated, three PCR products obtained with potato gene primers and all the six PCR products obtained with tomato gene primers and with degenerate primer pairs were sequenced. The DNA sequences were between 80 % and 100 % identical to the DNA sequences of corresponding genes in the database (Table 3-2). The variation occurred most frequently at the 3rd position of a codon followed by the 1st position while mutation at the 2nd positions was relatively less frequent. The *Pgk* gene is as an example for which a sequence was not available either in potato or in tomato. Degenerate primers were derived from consensus sequences from four different species (tobacco, Arabidopsis, spinach and wheat) and used to amplify the homologous segment in potato (see Sect. 3.1.1). A single PCR product of *Pgk* gene was obtained

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Table 3-2 Sequence similarity between the PCR products from potato and the corresponding reference sequences

Reference cDNA	Accession Number	Number of nucleotides compared	Similarity on nucleotide level (%)	Number of mismatches within a codon		
				1st	2nd	3rd
STP23 of potato [a]	D00520	660	100	0	0	0
SUS3 of potato [b]	U24088	710	98.5	1	0	5
SSSIII of potato [c]	X94400	333	100	0	0	0
GLO of tomato [d]	X92888	118	96.6	0	1	3
ENO of tomato [e]	X58108	334	97.0	2	1	7
NDPK of tomato [f]	X75324	390	94.4	5	2	15
PGK of tobacco [g]	Z48977	428	95.6	3	1	15
RCA of tomato [h]	AF037361	278	98.5	1	1	2
ANT of (I)	X65549	428	81.5	22	7	50
Arabidopsis [i] (II)		549	82.7	18	2	75

[a] STP23 = α-Glucan phosphorylase
[b] SUS3 = Sucrose synthase 3
[c] SSSIII = soluble starch synthase III
[d] GLO = Glycolate oxidase
[e] ENO = Enolase
[f] NDPK = Nucleoside diphosphate kinase
[g] PGK = phosphoglycerate kinase
[h] RAC = Rubisco activase
[i] ANT = adenylate transporter (two PCR fragments I and II were sequenced)

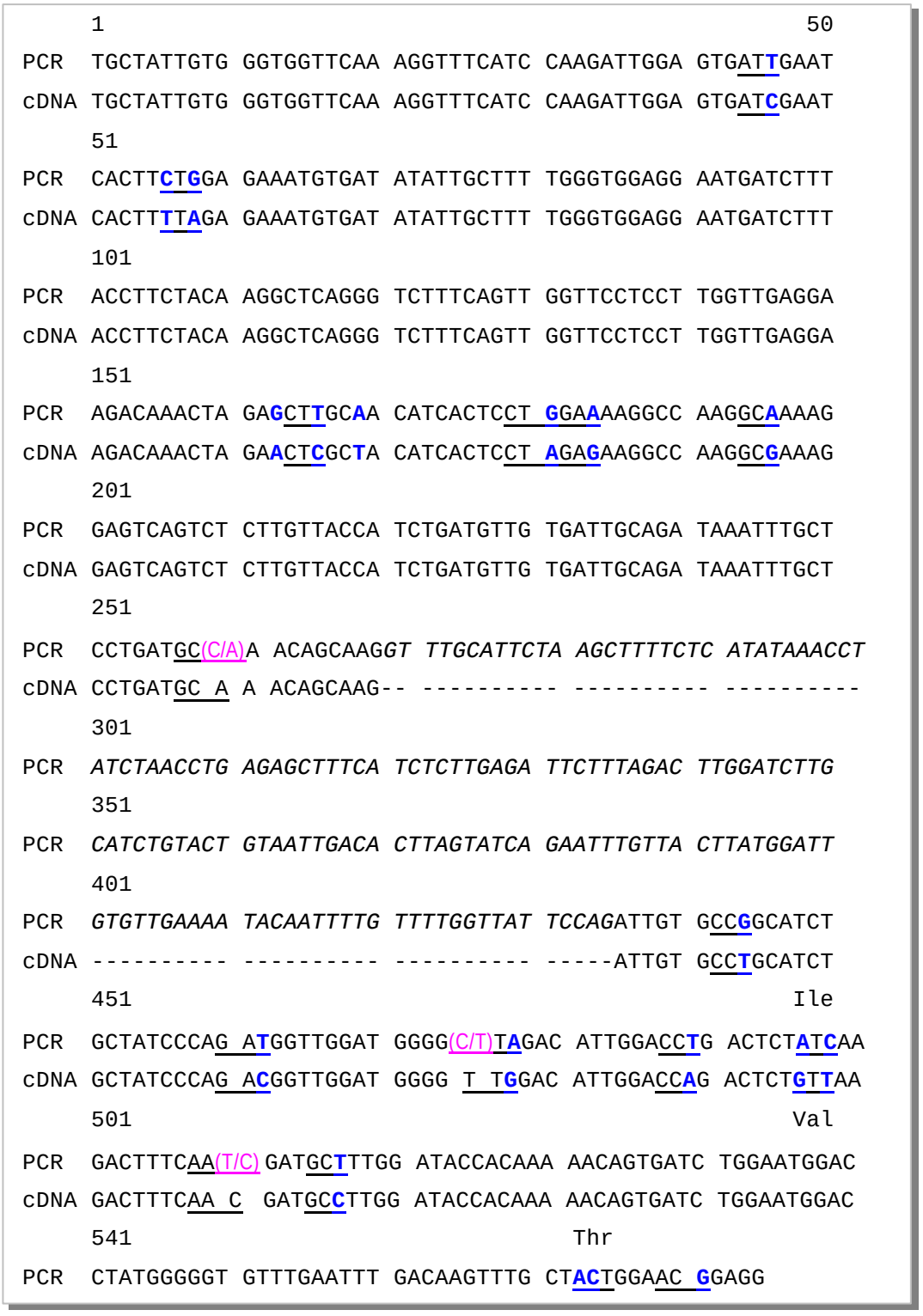


Figure 3-4 Comparison of the nucleotide sequence between potato PCR product of *Pgk* gene and one of the reference cDNA sequences of the gene (tobacco). Different nucleotides are highlighted in bold and the corresponding triplet codon is underlined. The nucleotide sequence of the intron is in italics. The alternative nucleotides at the same positions of the two alleles (SNP) are shown in parentheses. The three-letter code of amino acids was given for the non-synonymous changes.

(continued from page 58)

using genomic DNA of T710 (parent line of population LH) and the detailed sequence comparison between the PCR products and one of the reference gene sequences from tobacco is shown in Figure 3-4 (on page 59). Several features were revealed from the sequence comparison. First, the PCR product of the *Pgk* gene showed 95.6% similarity at the nucleotide level to the tobacco reference sequence. Most of the variations were point mutations and occurred at the third position of a triplet codon without changing the encoded amino acid. The only exceptions are at the positions 496-498 and 583-585. The mutations at these positions resulted in the changes of the coded amino acid from valine to isoleucine and from valine to threonine, respectively (Figure 3-4, on page 59). Second, sequence comparison of the PCR product of *Pgk* gene with the cDNA of tobacco revealed at position 268 a 167 bp intron with the characteristic feature of GT and AG motifs at each of the borders. Furthermore, sequencing from purified PCR products of the *Pgk* gene revealed directly three single nucleotide polymorphisms (SNPs) displaying a heterozygous nucleotides at positions 259, 475, and 510. Sequencing of all other PCR product revealed similar characteristics (see Section 7. Appendix)

3.1.5 An STS approach detected similar map positions as revealed by RFLP

Of the genes mapped, seven genes (*Sps ,Dbe, Sut1, Pha1,Pha2, Hxk, GluB*) were mapped by CAPS as well as by RFLP using different mapping populations (Table 3-3). The RFLP assays were previously performed by the lab of C. Gebhardt using the cloned genes as probes with the exception of *Dbe,* for which PCR product was used for RFLP mapping. In all seven cases, both marker assays identified similar loci. The case of *Pha*1 can be particularly mentioned. This gene identified two loci on LG III and LG VI by the RFLP assay with

Table 3-3 CAPS markers of seven genes localised to similar map positions as mapped by RFLP

		Mapping approach and population	
Gene	Linkage group	CAPS	RFLP
Hxk	III	K31	F1840
Sps	VII	LH	F1840
Dbe	XI	K31	LH
Pha1	III, VI	K31	F1840
Pha2	VII	LH	BC916[2]
Sut1	XI	K31	BC916[2]
GluB	I	K31	BC916[2]

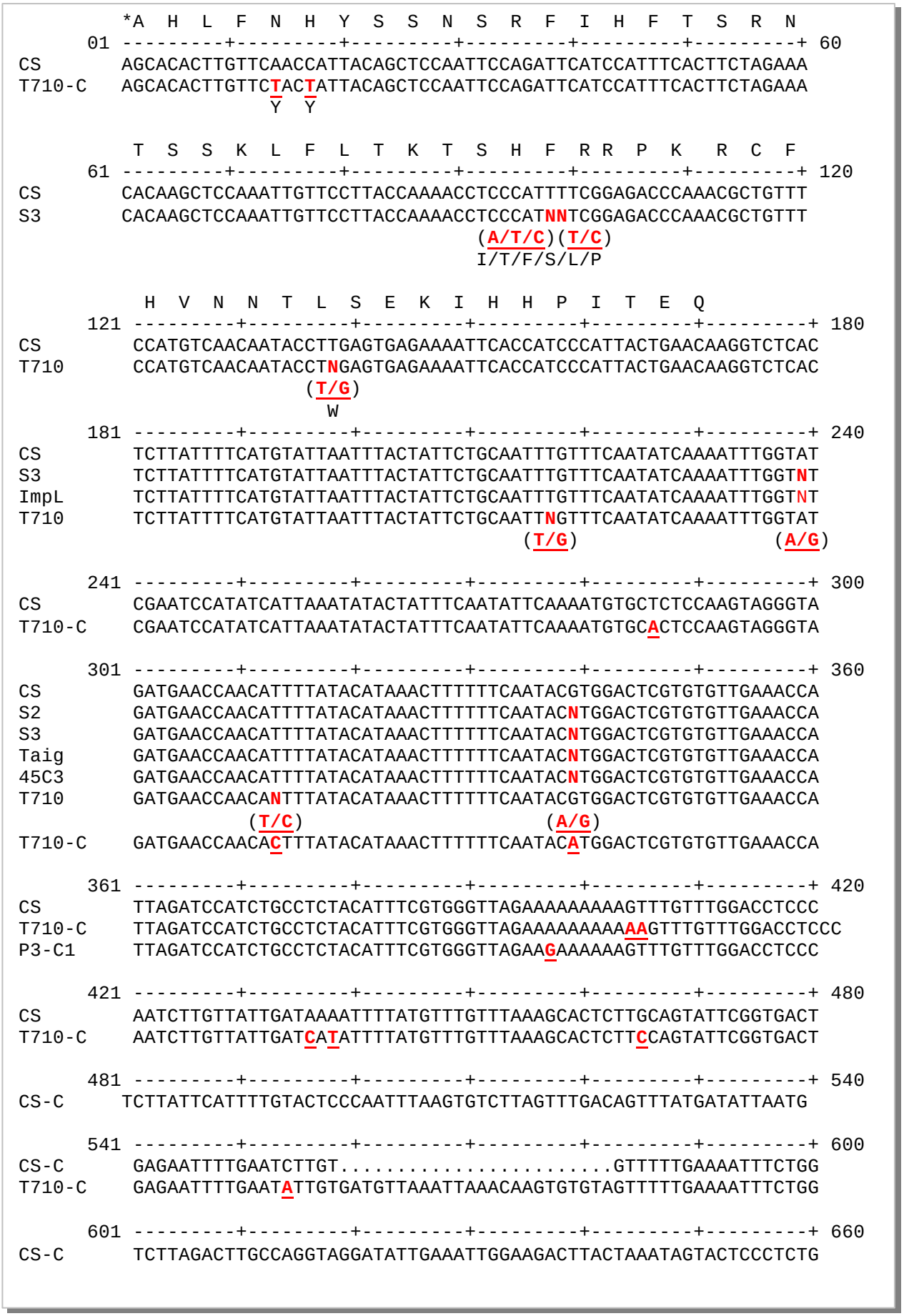
restriction enzyme *Taq*I using population F1840. These two loci were also uncovered by CAPS using population K31 in combination with *Taq*I.

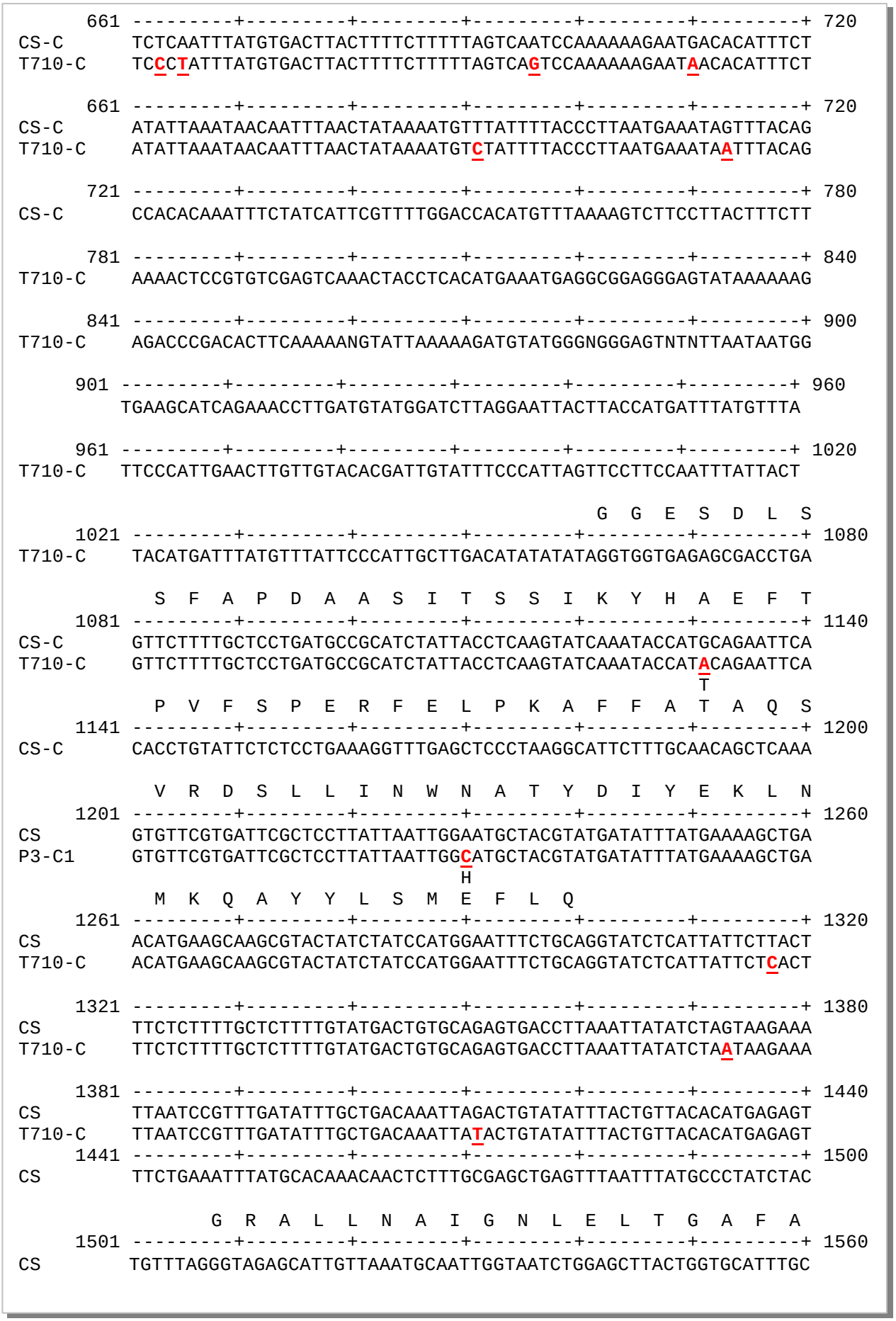
3.1.6 Overview of the potato function map

The current potato function map consists of 66 genes (including previously mapped genes) corresponding to 82 loci distributed over all 12 chromosomes (Table 3-1, page 51-55, Figure 3-3, page 57). 56 genes are directly or potentially involved in carbohydrate metabolism and transport of which 16 are involved in starch synthesis and degradation, 9 in sucrose metabolism, 8 in transport, 8 in the Calvin cycle and photorespiration, 8 in glycolysis and the oxidative pentose phosphate pathway, and 7 in the TCA cycle. The majority of the genes were identified as a single locus, eight gene markers identified two loci (*AGPaseB, Inv*$_{ap}$, *Pha1, Glo, Ppc, Fum1, Me, Ppa1*) and four gene markers detected three loci (*AGPaseS, rbcS, Ppe, Ldh2*).

3.2 Identification of single nucleotide polymorphisms (SNPs) in *Stp*23

14 potato lines with different genetic backgrounds and ploidy levels were selected to detect SNPs in *Stp*23 based on sequencing of PCR products. For all these lines the PCR products of *Stp*23 amplified with the primer pair shown in table 3-1 (on page 51-55) were bi-directionally sequenced. Apart from direct sequencing of the PCR products from all these lines, three lines P3, P38 and T710 were additionally cloned into the pGEM®-T vector prior to sequencing. One clone of each line was sequenced except P3 for which three clones were sequenced revealing two different alleles. The sequence comparison of all lines is shown in Figure 3-5 (on page 62-64). Sequence data obtained from PCR products and cloned fragments had readable sequences of up to 500-800 bp and 900-1100 bp, respectively, from each end. Analysis of sequences obtained from the cloned fragments revealed overlaps between the sequences of both directions, thus allowing the determination of the complete sequence of the PCR product. The PCR fragment had 2161 bases in length containing three introns with 891, 206 and 392 base pairs, respectively. For SNP identification, the low quality sequence of 360 bases in the overlapping region was excluded, only the high quality sequence region of 1801 bases was examined and compared to identify candidate SNPs. Of the nucleotide sequence examined, 653 bases are exonic while 1148 bases were intronic. ***(continue to page 64)***


*A H L F N H Y S S N S R F I H F T S R N
01 ---------+---------+---------+---------+---------+---------+ 60
CS AGCACACTTGTTCAACCATTACAGCTCCAATTCCAGATTCATCCATTTCACTTCTAGAAA
T710-C AGCACACTTGTTCTACTATTACAGCTCCAATTCCAGATTCATCCATTTCACTTCTAGAAA
Y Y
T S S K L F L T K T S H F R R P K R C F
61 ---------+---------+---------+---------+---------+---------+ 120
CS CACAAGCTCCAAATTGTTCCTTACCAAAACCTCCCATTTTCGGAGACCCAAACGCTGTTT
S3 CACAAGCTCCAAATTGTTCCTTACCAAAACCTCCCATNNTCGGAGACCCAAACGCTGTTT
(A/T/C)(T/C)
I/T/F/S/L/P
H V N N T L S E K I H H P I T E Q
121 ---------+---------+---------+---------+---------+---------+ 180
CS CCATGTCAACAATACCTTGAGTGAGAAAATTCACCATCCCATTACTGAACAAGGTCTCAC
T710 CCATGTCAACAATACCTNGAGTGAGAAAATTCACCATCCCATTACTGAACAAGGTCTCAC
(T/G)
W
181 ---------+---------+---------+---------+---------+---------+ 240
CS TCTTATTTTCATGTATTAATTTACTATTCTGCAATTTGTTTCAATATCAAAATTTGGTAT
S3 TCTTATTTTCATGTATTAATTTACTATTCTGCAATTTGTTTCAATATCAAAATTTGGTNT
ImpL TCTTATTTTCATGTATTAATTTACTATTCTGCAATTTGTTTCAATATCAAAATTTGGTNT
T710 TCTTATTTTCATGTATTAATTTACTATTCTGCAATTNGTTTCAATATCAAAATTTGGTAT
(T/G) (A/G)
241 ---------+---------+---------+---------+---------+---------+ 300
CS CGAATCCATATCATTAAATATACTATTTCAATATTCAAAATGTGCTCTCCAAGTAGGGTA
T710-C CGAATCCATATCATTAAATATACTATTTCAATATTCAAAATGTGCACTCCAAGTAGGGTA
301 ---------+---------+---------+---------+---------+---------+ 360
CS GATGAACCAACATTTTATACATAAACTTTTTTCAATACGTGGACTCGTGTGTTGAAACCA
S2 GATGAACCAACATTTTATACATAAACTTTTTTCAATACNTGGACTCGTGTGTTGAAACCA
S3 GATGAACCAACATTTTATACATAAACTTTTTTCAATACNTGGACTCGTGTGTTGAAACCA
Taig GATGAACCAACATTTTATACATAAACTTTTTTCAATACNTGGACTCGTGTGTTGAAACCA
45C3 GATGAACCAACATTTTATACATAAACTTTTTTCAATACNTGGACTCGTGTGTTGAAACCA
T710 GATGAACCAACANTTTATACATAAACTTTTTTCAATACGTGGACTCGTGTGTTGAAACCA
(T/C) (A/G)
T710-C GATGAACCAACACTTTATACATAAACTTTTTTCAATACATGGACTCGTGTGTTGAAACCA
361 ---------+---------+---------+---------+---------+---------+ 420
CS TTAGATCCATCTGCCTCTACATTTCGTGGGTTAGAAAAAAAAAGTTTGTTTGGACCTCCC
T710-C TTAGATCCATCTGCCTCTACATTTCGTGGGTTAGAAAAAAAAAAAGTTTGTTTGGACCTCCC
P3-C1 TTAGATCCATCTGCCTCTACATTTCGTGGGTTAGAAGAAAAAAGTTTGTTTGGACCTCCC
421 ---------+---------+---------+---------+---------+---------+ 480
CS AATCTTGTTATTGATAAAATTTTATGTTTGTTTAAAGCACTCTTGCAGTATTCGGTGACT
T710-C AATCTTGTTATTGATCATATTTTATGTTTGTTTAAAGCACTCTTCCAGTATTCGGTGACT
481 ---------+---------+---------+---------+---------+---------+ 540
CS-C TCTTATTCATTTTGTACTCCCAATTTAAGTGTCTTAGTTTGACAGTTTATGATATTAATG
541 ---------+---------+---------+---------+---------+---------+ 600
CS-C GAGAATTTTGAATCTTGT.....GTTTTTGAAAATTTCTGG
T710-C GAGAATTTTGAATATTGTGATGTTAAATTAAACAAGTGTGTAGTTTTTGAAAATTTCTGG
601 ---------+---------+---------+---------+---------+---------+ 660
CS-C TCTTAGACTTGCCAGGTAGGATATTGAAATTGGAAGACTTACTAAATAGTACTCCCTCTG


661 ---------+---------+---------+---------+---------+---------+ 720
CS-C TCTCAATTTATGTGACTTACTTTTCTTTTTAGTCAATCCAAAAAAGAATGACACATTTCT
T710-C TCCCTATTTATGTGACTTACTTTTCTTTTTAGTCAGTCCAAAAAAGAATAACACATTTCT
661 ---------+---------+---------+---------+---------+---------+ 720
CS-C ATATTAAATAACAATTTAACTATAAAATGTTTATTTTACCCTTAATGAAATAGTTTACAG
T710-C ATATTAAATAACAATTTAACTATAAAATGTCTATTTTACCCTTAATGAAATAATTTACAG
721 ---------+---------+---------+---------+---------+---------+ 780
CS-C CCACACAAATTTCTATCATTCGTTTTGGACCACATGTTTAAAAGTCTTCCTTACTTTCTT
781 ---------+---------+---------+---------+---------+---------+ 840
T710-C AAAACTCCGTGTCGAGTCAAACTACCTCACATGAAATGAGGCGGAGGGAGTATAAAAAAG
841 ---------+---------+---------+---------+---------+---------+ 900
T710-C AGACCCGACACTTCAAAAANGTATTAAAAAGATGTATGGGNGGGAGTNTNTTAATAATGG
901 ---------+---------+---------+---------+---------+---------+ 960
TGAAGCATCAGAAACCTTGATGTATGGATCTTAGGAATTACTTACCATGATTTATGTTTA
961 ---------+---------+---------+---------+---------+---------+ 1020
T710-C TTCCCATTGAACTTGTTGTACACGATTGTATTTCCCATTAGTTCCTTCCAATTTATTACT
G G E S D L S
1021 ---------+---------+---------+---------+---------+---------+ 1080
T710-C TACATGATTTATGTTTATTCCCATTGCTTGACATATATATAGGTGGTGAGAGCGACCTGA
S F A P D A A S I T S S I K Y H A E F T
1081 ---------+---------+---------+---------+---------+---------+ 1140
CS-C GTTCTTTTGCTCCTGATGCCGCATCTATTACCTCAAGTATCAAATACCATGCAGAATTCA
T710-C GTTCTTTTGCTCCTGATGCCGCATCTATTACCTCAAGTATCAAATACCATACAGAATTCA
T
P V F S P E R F E L P K A F F A T A Q S
1141 ---------+---------+---------+---------+---------+---------+ 1200
CS-C CACCTGTATTCTCTCCTGAAAGGTTTGAGCTCCCTAAGGCATTCTTTGCAACAGCTCAAA
V R D S L L I N W N A T Y D I Y E K L N
1201 ---------+---------+---------+---------+---------+---------+ 1260
CS GTGTTCGTGATTCGCTCCTTATTAATTGGAATGCTACGTATGATATTTATGAAAAGCTGA
P3-C1 GTGTTCGTGATTCGCTCCTTATTAATTGGCATGCTACGTATGATATTTATGAAAAGCTGA
H
M K Q A Y Y L S M E F L Q
1261 ---------+---------+---------+---------+---------+---------+ 1320
CS ACATGAAGCAAGCGTACTATCTATCCATGGAATTTCTGCAGGTATCTCATTATTCTTACT
T710-C ACATGAAGCAAGCGTACTATCTATCCATGGAATTTCTGCAGGTATCTCATTATTCTCACT
1321 ---------+---------+---------+---------+---------+---------+ 1380
CS TTCTCTTTTGCTCTTTTGTATGACTGTGCAGAGTGACCTTAAATTATATCTAGTAAGAAA
T710-C TTCTCTTTTGCTCTTTTGTATGACTGTGCAGAGTGACCTTAAATTATATCTAATAAGAAA
1381 ---------+---------+---------+---------+---------+---------+ 1440
CS TTAATCCGTTTGATATTTGCTGACAAATTAGACTGTATATTTACTGTTACACATGAGAGT
T710-C TTAATCCGTTTGATATTTGCTGACAAATTATACTGTATATTTACTGTTACACATGAGAGT
1441 ---------+---------+---------+---------+---------+---------+ 1500
CS TTCTGAAATTTATGCACAAACAACTCTTTGCGAGCTGAGTTTAATTTATGCCCTATCTAC
G R A L L N A I G N L E L T G A F A
1501 ---------+---------+---------+---------+---------+---------+ 1560
CS TGTTTAGGGTAGAGCATTGTTAAATGCAATTGGTAATCTGGAGCTTACTGGTGCATTTGC

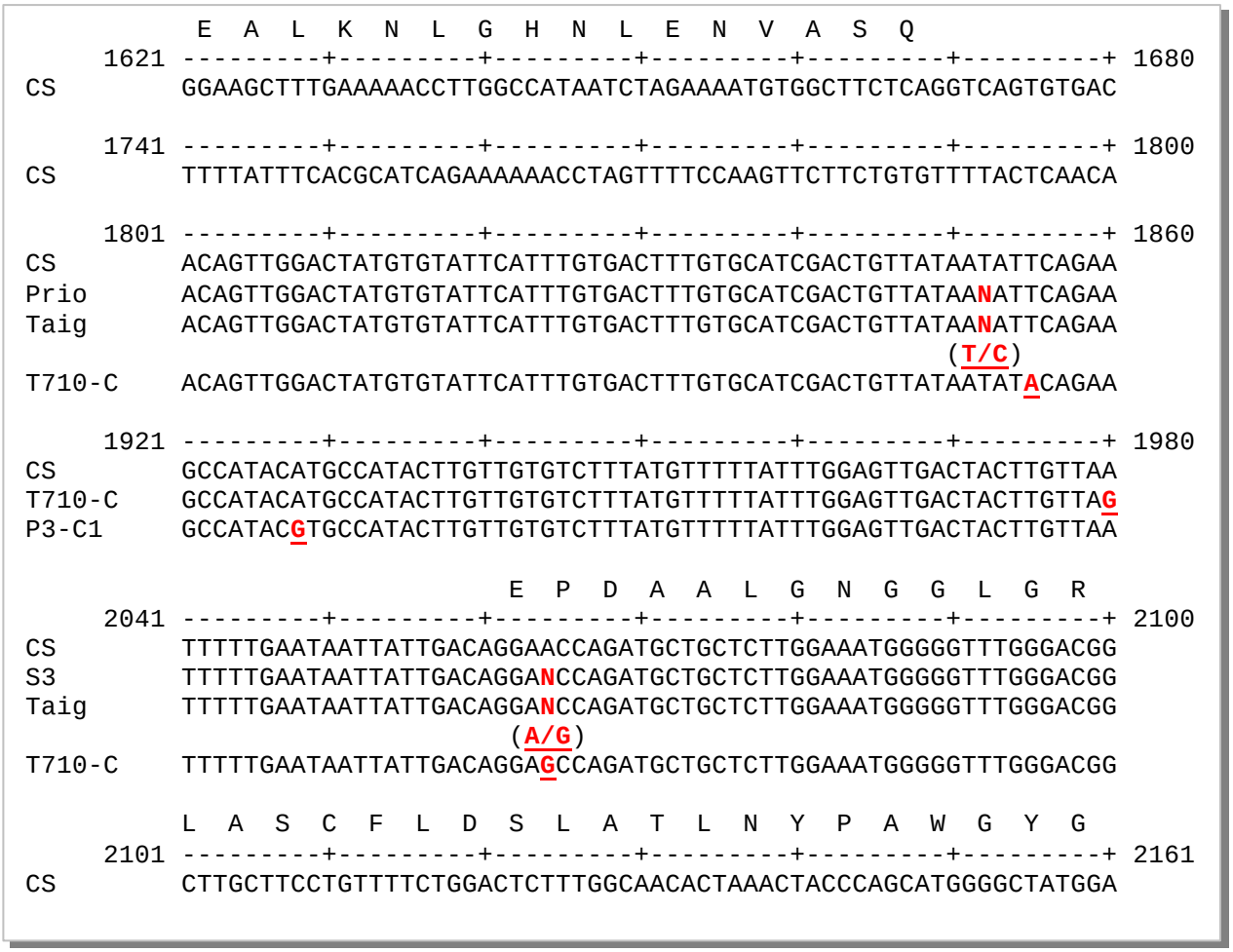


Figure 3-5 The alignment of partial sequences of *Stp*23 showing the features of single nucleotide polymorphisms between different alleles of different potato lines. Potential SNPs were highlighted in bold (red) and underlined. For simplicity, only the sequences differing from the majority were aligned with the majority consensus sequence (CS). Suffix '-C' indicates that sequence data was based on a cloned PCR product, otherwise, sequence data directly from a PCR product was used. '*' Single-letter code for amino acids and substitutions of amino acids are shown above and below the corresponding exonic nucleotide sequence. The dotted line indicates a gap in the sequence relative to the compared sequence.

(continued from page 61)

Sequence data obtained from PCR products was first examined for different bases at single positions, then sequences were aligned and each vertical slice of the resulting assembly was examined for disagreement. Any such slice was considered a candidate SNP. Sequencing directly from PCR products allowed the identification of multiple or alternative bases at the same positions within individuals. 8 bi-allelic SNPs were detected in diploid and tetraploid potato (Figure 3-5, Figure 3-6, on page 65). In one instance, a tri-allelic SNP was found in tetraploid potato line S3 (Figure 3-6, on page 65). Sequence comparison between different lines revealed single nucleotide differences between the alleles which compose the potential SNP at that position. In three instances, the alternative base observed in different lines was

also observed within individual lines at the same position (position 314, 340 and 2065, Figure 3-5, on page 62-64). In all, 31 positions were detected which could potentially be SNPs for *Stp*23 in the region examined, representing 1.7 % (31/1801) on average. 8 were in the coding region (cSNPs) whereas 23 were in introns with nucleotide diversity of 1.2% (8/653) and 2% (23/1148), respectively. Of the 8 potential cSNPs, 6 resulted in non-synonymous changes. One allele of T710, a wild species (*S. chacoense*), showed remarkably high variability as compared to the others with 20 disagreements with consensus being observed in the allele. When considering transitions versus transversions of SNPs, the frequencies of the two types

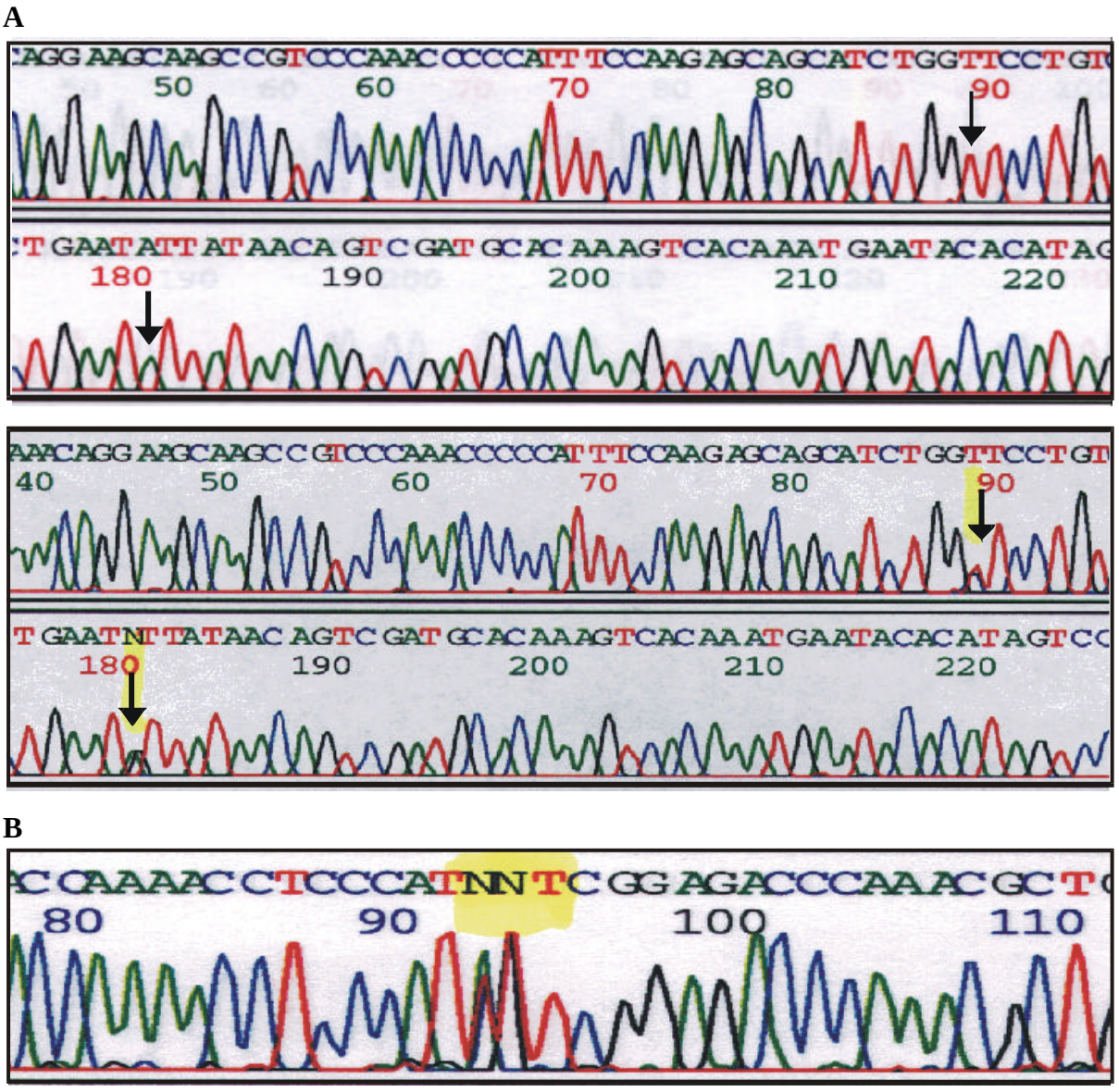


Figure 3-6 Partial electropherogram of nucleotide sequence data from a PCR product of gene *Stp*23 showing the different types of SNP. (A) bi-allelic SNP (indicated by the arrows): Homozygote (potato line Hands, upper panel) T vs. heterozygote (potato line Taiga, lower panel) C/T at position 89 and A vs. G/A at position 181; (B) tri-allelic SNP (marked yellow) in tetraploid potato line S3 at position 93.

of SNP were different. The majority of SNPs (19) involved a transition (C/T or G/A) while the remaining SNPs were transversions (C/A or G/T, C/G or G/C, T/A or A/T).

3.3 Molecular tagging of QTL in tetraploid potato by fAFLP markers

3.3.1 Genotyping of the Z3 family by an automated fAFLP system

3.3.1.1 Screening primer combinations for fAFLP

Efficient genotyping by AFLP depends on the restriction enzymes and primer combinations used. On one hand, restriction enzymes that generate fragments evenly distributed over a size of 50-500 bp facilitate data collection, thus it is highly desirable for AFLP marker generation. On the other hand, the degree of polymorphism between individuals may be variable with different primer combinations. Generation of a high degree of polymorphism between individuals could maximise the potential use of primer pairs in genotyping. Therefore, the first step in the study has been a comparison of the efficiency of different primer sets and screening of primer combinations. To do this, 12 individuals were randomly selected from population Z3 to be tested with different primer sets and primer combinations for polymorphisms. The first round of screening was carried out to determine the primer sets from four candidate pairs of restriction enzymes including *Eco*RI/*Mse*I, *Hind*III/*Mse*I, *Eco*RI/*Taq*I and *Hind*III/*Taq*I.

Of the four primer sets tested, *Hind*III/*Mse*I produced less informative fragments than either *Hind*III/*Taq*I or *Eco*RI/*Taq*I, while the latter two generated larger (data not shown) and more fragments than *Eco*RI/*Mse*I (Table 3-4, 3-5, on page 67). However, the number of polymorphic fragments did not increase. The *Eco*RI/*Mse*I primer set, which generated more informative and more evenly distributed AFLP fragments within the size range of 50-500 bp, was selected and more primer combinations of this primer set were tested for the efficiency of generating informative fragments (Table 3-5, on page 67). A total of 29 primer combinations were further tested for primer set *Eco*RI/*Mse*I (Table 3-5). 15 primer pairs generated more than 25 informative fragments. Based on the number of informative fragments generated and the selective nucleotide diversity of the primers, 7 of them were selected for genotyping all the individuals of breeding population Z3. The 7 primer combinations were E-aca/M-cat, E-act/M-cag, E-act/M-cta, E-aag/M-ctg, E-acg/M-cac, E-

agg/M-caa and E-acc/M-cta, which were coded as B1, B2, B3, G1, G2, G3 and Y, respectively, for simplicity.

Table 3-4 Number of polymorphic versus total AFLP fragments generated by primer sets *Eco*RI/*Taq*I and *Hind*III/*Taq*I

	T-gta	T-gat	T-atg	T-agt	M-acg	M-aat
H-agt		3/95		3/87	19/149	10/161
H-aga			22/129	18/208	5/98	15/177
H-act	39/209		20/216	20/205	15/130	12/197
H-ata	22/121	15/103	6/152	8/139	12/96	5/90
E-act	12/166	7/156				
E-aag	16/124	18/165				

Table 3-5 Number of polymorphic versus total AFLP fragments generated by *Eco*RI/*Mse*I primer sets*

	M-ctg	M-cat	M-cag	M-cac	M-cta	M-ctc	M-caa	M-ctt
E-aag	**31/115**		9/96	24/115	5/96	15/101		
E-acg			29/126	**27/92**	19/90	14/90		8/94
E-agg			28/101		8/106	25/106	**38/129**	
E-aca	6/57	**26/151**		25/103				
E-act		19/154	**29/109**	27128	**27/117**			
E-aac			10/110					6/99
E-agc					18/135	15/104		
E-acc			29/105		**40/108**	23/117	40/149	

*Primer combinations used for genotyping the Z3 population are highlighted in bold

3.3.1.2 fAFLP genotyping and data collection

Of the seven primer combinations selected for genotyping, selective primers E-aca and E-act were fluorescently labelled with FAM (blue), E-agg, E-acg and E-aag were labelled with JOE (green) and E-acc with TAMRA (yellow). Selectively amplified products labelled with different fluorescent dyes were alternatively loaded and resolved by electrophoresis on a 377 DNA sequencer. An example of a gel image on the 377 DNA sequencer is shown in Figure 3-7 (on page 68). Figure 3-8A (on page 69) shows fragment patterns ***(continue to page 69)***

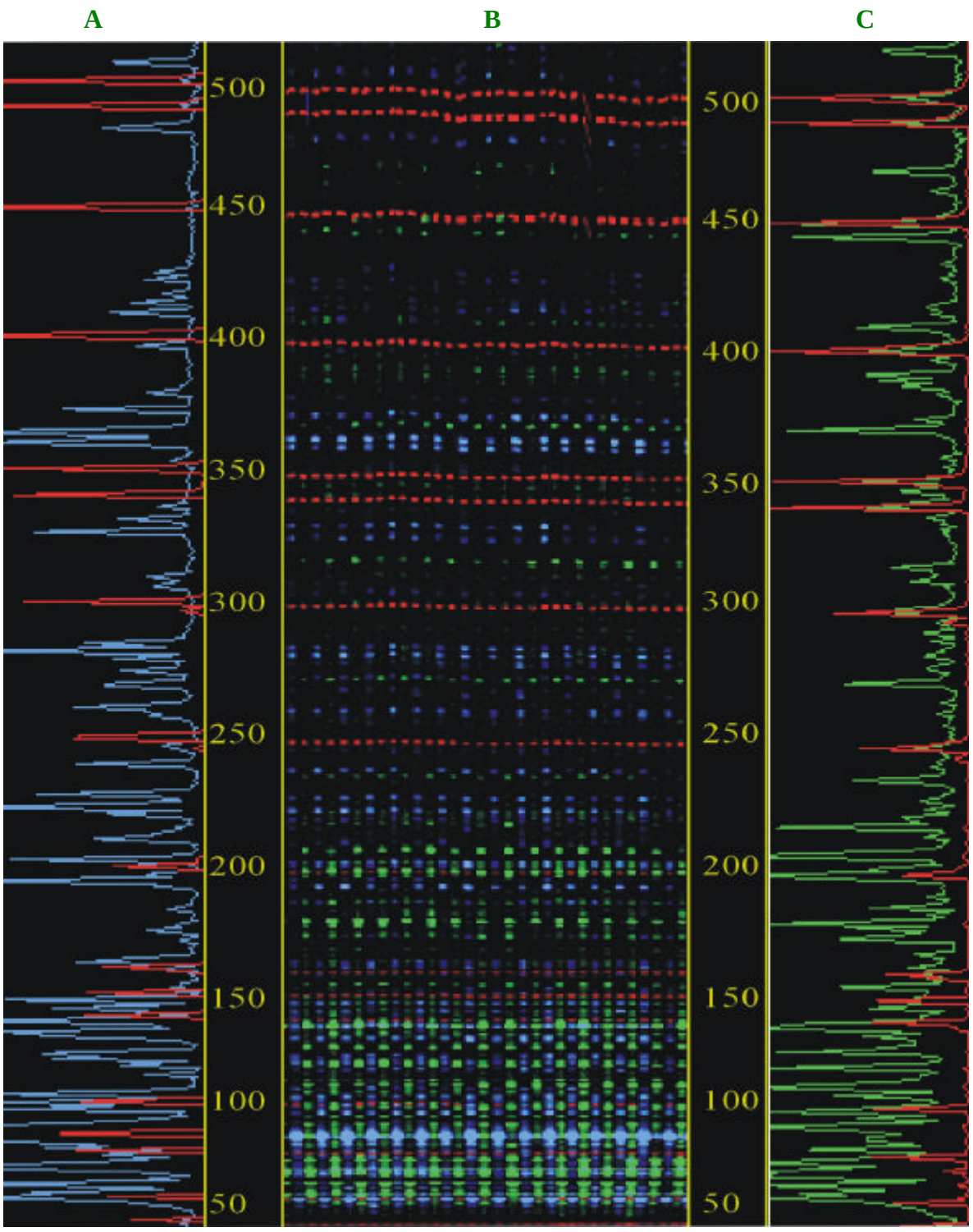


Figure 3-7 Electropherogram and gel view of fAFLP samples run on the ABI PRISMÔ 377 DNA sequencer. Panel **B** shows gel image of fAFLP fragments generated with fluorescence-labelled primer pairs E-act/M-cag (blue) and E-aag/M-ctg (green). Red fragments are internal size standards with size in bp marked on the sides. Panel **A** and **C** show GeneScan®-generated electropherograms of two samples from the gel image.

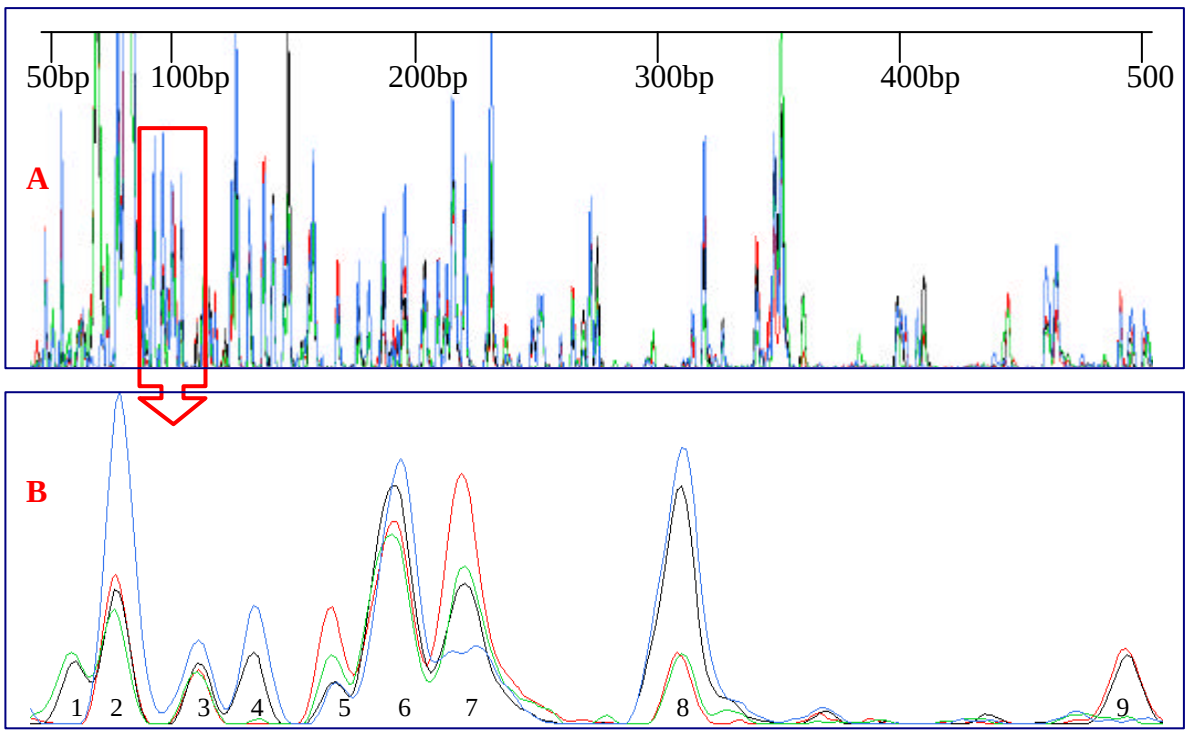


Figure 3-8 GeneScanÒ-generated fAFLP electropherogram of four samples displayed in different colours. Primer pair used: E-act/M-cag, **A**: overview of the fragment pattern over the range 50-500 bp; **B**: the enlarged details of fragments (1-9) around 100 bp showing homomorphic fragments (2, 3, 5, 6, 7, 8) and polymorphic fragments (1, 4, 9).

(continued from page 67)

of four individuals distinguished by four colours in one panel and the Figure 3-8B shows partially enlarged details of the fragments displaying polymorphic and homomorphic fragment peaks and peak height variations. All the information in terms of both size and intensity of each fragment was collected and processed using the computer GeneScan® software. Prior to automated genotyping using the software program Genotyper™, the overall intensity of the fragments from each sample was proportionately adjusted by referring to the intensity of a pre-set reference sample. The variation of fragment intensity caused by environmental factors was therefore minimised. The final data files containing information of fragment size in base pairs and fragment intensity in peak height were created using computer software Genotyper™ and exported into an *Excel* file format which was subsequently used for QTL analysis.

Characteristics of the fAFLP data

The number of fragments within the size range of 50-500 bp generated with the 7 primer combinations is shown in Table 3-6 (on page 70). Between 90-150 fragments were

individually amplified with each of the 7 AFLP primer combinations. Of the fragments generated, 185 and 152 were derived from S1 and S3, respectively, while 469 descended from both parents (Table 3-6). In total, 806 different-sized fragments were scored with respect to their peak height which was detected and quantified automatically by GeneScan®. More than 200 fragments were estimated to be polymorphic based on the pre-screen data (Table 3-5, on page 67).

Table 3-6 Number of fAFLP markers generated by seven primer combinations in population Z3 and their origins

	S1	S3	Common	Total
B1	36	35	80	151
B2	24	31	53	108
B3	19	24	74	117
G1	35	19	61	115
G2	27	7	58	92
G3	27	22	80	129
Y	17	14	63	94
Total	185	152	469	806

B1=E-aca/M-cat, B2=E-act/M-cag , B3=E-act/M-cta
G1=E-aag/M-ctg , G2=E-acg/M-cac , G3=E-agg/M-caa
Y=E-acc/M-cta

For a given locus in the tetraploid population, the allele dosage ranged from nulliplex to quadruplex which may contribute to the variation of the phenotypic traits, while the fragment intensity quantified by the GeneScan® software as fragment peak height reflected, to some extent, allele dosage. Thus, quantitatively (scored as peak height) and qualitatively (scored as presence and absence) scored fragments were both subjected to QTL analysis using appropriate statistical tools.

3.3.2 Analysis of phenotypic traits and phenotypic data*

150-200 individuals of population Z3 were evaluated for five phenotypic traits including tuber starch content (TSC), reducing sugar content (RSC), foliage earliness (FE) development (DEV) and tuber yield (TY). The phenotypic data for 1997, which was created from scoring a single plant individual (no replication), was not subjected to analysis separately but was merged with the data of 1998 for which the scores resulted from 5 different plants of each clone (Strahwald, 1999, personal communication). The combined data were used for further analysis. Heritability and correlation coefficients of the traits were calculated and are shown in Table 3-7 based on 1998 data. ***(continue to page 72)***

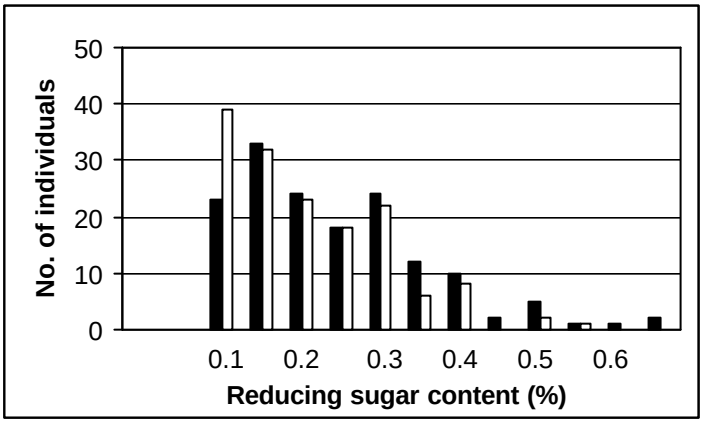
*Phenotypic data were evaluated in field trials at two locations (Gransebieth and Windeby) over three years (1997, 1998, and 1999) by the potato breeding company SaKa-Ragis (Dr. Josef Strahwald). They are summarized here because the phenotypic data was an essential part of the QTL analysis.

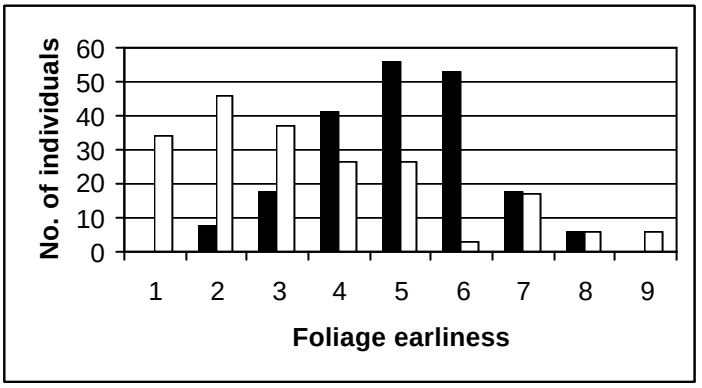
Table 3-7 Heritability and correlation coefficient of the phenotypic traits evaluated*

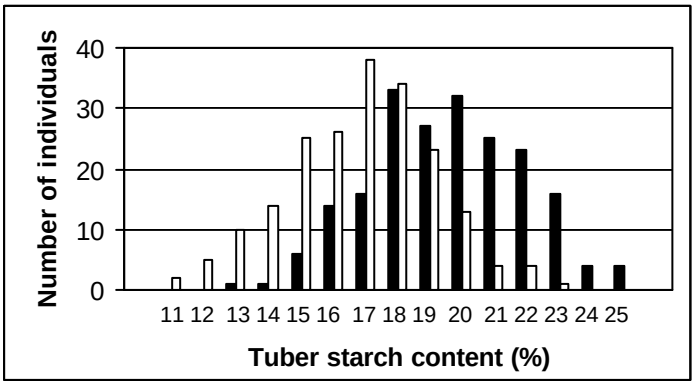
	H^2	r		
		DEV	FE	TSC
FE	0.65	0.27		
TSC	0.59	0.17	0.49	
RSC	0.62		-0.26	-0.62
TY	0.14		-0.18	
DEV	0.32			

FE= foliage earliness
TSC= tuber starch content
RSC= reducing sugar content
TY= tuber yield
DEV= development
H^2= broad-sense heritability
r= correlation coefficient
significance level: 0.01
Correlation coefficients, which were not significant at this level, are not shown in the table.

Figure 3-9 Frequency distribution of phenotypic scores for
A: reducing sugar content (% fresh weight)
B: foliage earliness (1= very early, 9= very late);
C: tuber starch content (% fresh weight)
Based on the data collected from trial 1998 at two locations, Gransebieth (blank column) and Windeby (black column) using population Z3.
200 individuals were evaluated for foliage earliness and tuber starch content, whereas 150 individuals were assessed for reducing sugar content.







A
B
C

(continued from page 70)

Of the five traits investigated, significant correlations were revealed among the traits (Table 3-7, on page 71). It should be noted that TSC, RSC and FE showed significant correlations and relatively high correlation coefficients between each other. TSC had a significant negative correlation with RSC (r=-0.62, $P<0.01$) and positive correlation with FE (r=0.49, $P<0.01$, the higher the TSC, the later the foliage matures). There are also significant correlations between RSC and FE (r=-0.26, $P<0.01$, the higher the reducing sugar content, the earlier the foliage matures).

Heritability of different traits was quite variable in the population studied. TSC, RSC and FE showed high heritability, while tuber yield and development had rather lower heritability (Table 3-7, on page 71). These observations indicated that the phenotypic variation observed for the former three traits resulted mainly from genetic variation, whereas, the latter two were largely caused by environmental factors. Therefore, further studies were only concentrated on the three high heritability traits TSC, RSC and FE.

Based on the method used for evaluating the three traits (see Sect 2.14), it is obvious that the phenotypic data for FE and RSC were categorical since they were based on or converted from a 1-9 reference standard scale. Thus both data sets are discontinuous. The data for TSC is quantitative, continuous and normally distributed (Figure 3-9C, on page 71) which was indicated by a Kolmogorov-Smirnov test ($p > 0.2$). TSC ranged from 13.6 % to 25.8 % in Windeby and 7.1 % to 22.4 % in Gransebieth with mean values of 20.21% and 16.34 %, and standard deviation of 2.36 and 2.29, respectively. The phenotypic distribution of the three traits are graphically exhibited in histograms of Figure 3-9 (on page 71). The distributions were consistent with the assumed polygenic inheritance of the traits considered. According to the nature of the phenotypic data, parametric and non-parametric methods of statistical analysis were applied to detect marker-trait associations for TSC, RSC and FE.

3.3.3 Identification of QTL associated with fAFLP fragments

The QTL analysis was performed in collaboration with Dr. Schäfer-Pregl (MPIZ). The data collected from the two different locations were analysed to determine the significance and the amount of phenotypic variance explained by fAFLP markers. Statistical analysis as described in Sect. 2.15 was applied to identify the associations of phenotypic traits with fAFLP

(continue to page 74)

Table 3-8 fAFLP markers showing associations with QTL for TSC as detected by the t-test and regression analysis

					t-test				Regression analysis			
					Gransebieth		Windeby		Gransebieth		Windeby	
fAFLP marker	LG[a]	No -/+[b]	χ^2-test[c]	Ori.[d]	*P*[e]	R^{2f} (%)	*P*[e]	R^{2f} (%)	*P*	R^{2f} (%)	*P*	R^{2f} (%)
B3-409		138/51		S1	0.00017+	6.0	0.0018+	5.4	0.0075	3.9	0.0023	5.0
B1-213		158/27		S3	0.014-	4.4	0.0053-	5.6	0.011	3.4	0.032	2.5
B1-214		36/109	1:3	C	0.015-	3.6	0.0051-	4.7	0.0014	5.3	0.0047	4.2
B2-429		138/42		S3	0.025-	3.1	0.04-	2.8	0.02	3.0	0.017	3.2
G1-169		136/32		S3	0.0076-	3.2	0.014-	3.0	0.018	3.3	0.035	2.6
G1-435		116/51		S1	0.022+	3.1	0.0046+	4.5			0.018	3.3
B2-306		15/146	1:11	C	0.041-	3.5	0.026-	4.2			0.022	3.2
B1-174	V	21/174	1:11	C	0.045+	2.7	0.033+	1.5				
B3-134		143/40		S1	0.022+	1.6	0.017+	2.8				
B3-397		137/45		S1	0.0023+	5.1	0.041+	2.2				
B1-124		36/158		C	0.033-	2.1	0.026-	2.7				
G3-393	I	133/46		S1	0.010-	4.7	0.024-	3.2				
B1-244		137/43		S3	0.018-	3.6			0.009	3.8		
B2-428		57/122		C	0.011+	4.2			0.014	3.4		
B3-260	II	135/52		S1	0.011+	3.1			0.01	3.6		
G1-325		118/24		C	0.016+	3.3			0.024	2.7		
G2-187		49/137	1:3	C	0.0037-	4.2			0.0066	4.1		
G3-68		90/95		C	0.015-	3.2			0.024	2.7		
G3-101		75/110		C	0.0053-	4.1			0.027	2.6		
B1-316	III	67/119		C			0.0029-	4.7			0.0078	3.8
B2-168	V	104/77		S1			0.048+	2.2			0.043	2.3
B2-352		116/65		S1			0.01-	3.8			0.033	2.5
B3-121	IX	62/130		C			0.0035+	3.8	0.034	2.4	0.0003	6.9
G1-200		140/49		S1			0.032-	2.3			0.0094	3.5
G3-163		49/132	1:3	C			0.028-	2.5			0.032	2.5
G3-342	III	61/116		S3			0.043+	2.6			0.01	3.7
G1-156		20/171	1:11	C			0.045-	2.1	0.0066	3.8		
Y-167		57/97		S1	0.022-	3.3	0.043-	2.5				
B1-166	I	100/94	1:1	S3	0.0069-	3.7						
B2-76	V	90/95		C			0.0011-	5.7				
G3-490		49/122	1:3	C					0.002	5.2	0.022	2.9
B1-107		191		C					0.013	3.2	0.018	2.9
Gl-184		185		C					0.013	3.3	0.0022	5.0
G1-109		190		C					0.0006	6.0		
G1-324		82/93	1:1	S1					0.0049	4.2		
G3-437		48/111		C					0.006	4.5		
B2-173	XII	186		C							0.0021	5.0
B3-120		183		C							0.0057	4.1
G1-330		49/140	1:3	C							0.0017	5.1
G3-429		107/76		C							0.0058	4.1

[a] LG= linkage group, inferred from map positions of common fAFLP markers in the reference diploid mapping population K31.
[b] No. -/+, number of individuals analyzed showing absence/presence at the marker locus.
[c] 1:N, segregation type confirmed by goodness-of-fit test.
[d] Ori., origin of the marker with 'C' representing common marker from both parents.
[e] *P* values followed by plus or minus indicate the direction of the tagged QTL effects with '+' being increasing and '-' being decreasing TSC.
[f] R^2= R square value in percent indicating the proportion of variance explained by the marker genotype.

(continued from page 72)

markers. Briefly, parametric tests, a t-test and regression analysis, were applied to data of TSC, while the non-parametric Mann-Whitney U-test was used for association analysis between markers and traits for data of RSC and FE. The existence of a fAFLP-tagged QTL was accepted when the marker was consistently detected to be associated with one trait in both locations, or with two or more different traits at a threshold of $P<0.05$. Fragments for which association was detected in only one location with only one trait was considered of potential interest only when $P<0.01$. The fAFLP fragments, which meet the above criteria, are presented in the Sect. 3.3.3.1.

3.3.3.1 Association of fAFLP fragments with tuber starch content (TSC)

Association analysis using the t-test and linear regression resulted in a total of 40 fAFLP markers putatively associated with QTL for TSC with the threshold chosen. A nomenclature for fAFLP was adopted by starting with a primer combination code followed by the fragment size detected. The fAFLP markers, magnitude and direction of QTL effects are summarized in the order of their reproducibility in Table 3-8 (on page 73). Among the 40 fAFLP markers as identified by t-test and regression analysis, 5 markers (B3-409, B1-213, B1-214, B2-429, G1-169) were consistently detected at two locations and with two different tests; 3 markers (G3-490, B1-107, and G1-148) were repeatedly detected but only with regression analysis; 7 markers (G1-435, B2-306, B1-174, B3-134, B3-397, B1-124, and G3-393) were reproducible at two locations using the t-test but not detected or detected only at one location using regression analysis. 15 markers were reproducibly detected using both the t-test and regression analysis in one of the locations. Others were detected only in a single test. Of the 12 markers consistently detected at both locations using the t-test, markers B3-409, G1-435, B1-174, B3-134 and B3-397, which all originated from parent S1, showed a positive effect on TSC whereas other markers descending from both parents or from S3 only, showed negative effects on TSC. This observation suggested that the S1 allele at these marker loci was associated with an increase in TSC. These markers, by ANOVA, explained 1.5-6 % of the phenotypic variation individually. The most significant effect on TSC was associated with the marker B3-409. The allelic variation at this marker locus accounted for up to 6 % of the phenotypic variation. Given the broad-sense heritability of 59 % for TSC, this marker explained 10 % of the total genetic variance.

3.3.3.2 Association of fAFLP fragments with reducing sugar content (RSC) and foliage earliness (FE)

Due to the discontinuous nature of the phenotypic data for the both traits, the non-parametric Mann-Whitney U-test was applied to the analysis for the marker-trait association. The results were shown in Table 3-9.

fAFLP markers associated with RSC

Over two locations, a total of 25 markers were associated with RSC (Table 3-9). 11 of these markers were consistent across two locations at the 5% or 1% significance level, the others were detected at only one location at the 1% level. The majority of the markers (10) tagged alleles of the parent S3 which contributed to increased RSC. ***(continue to page 76)***

Table 3-9 fAFLP markers showing associations with QTL for RSC and FE as detected by the Mann-Whitney U-test

					Reducing sugar content				Foliage earliness			
					Gransebieth		Windeby		Gransebieth		Windeby	
fAFLP marker	LG[a]	No -/+[b]	χ^2-test[c]	Ori.[d]	*P*[e]	R^{2f} (%)	*P*[e]	R^{2f} (%)	*P*[e]	R^{2f} (%)	*P*[e]	R^{2f} (%)
B3-117		18/117		C	0.009-	3.9	0.0009-	12.6				
B3-413		40/96	1:3	C	0.019-	2.4	0.002-	5.7				
B3-69		75/70	1:1	S1	0.013-	3.7	0.027-	2.8				
B2-123		27/113	1:3	C	0.044+	1.8	0.017+	2.0				
B3-360		42/103	1:3	C	0.026+	3.4	0.021+	3.6				
G2-386		73/44		S3	0.003+	7.2	0.002+	5.2				
G3-102		9/128	1:11	C	0.034+	2.7	0.016+	3.3				
Y-452		40/91	1:3	C	0.037+	3.8	0.045+	3.1				
B1-330		94/47		S3	0.009+	3.6	0.006+	6.1	0.041-	1.6		
B3-215	V	84/60		S3	0.0004+	6.6	0.0001+	11.3	0.043+	1.9		
G3-79		82/58		S3	0.005+	7.8	0.029+	2.9	0.005+	4.6	0.001+	5.2
B1-158	II	42/149	1:3	C					0.043+	2.8	0.027+	2.3
B1-225		147/33		S3					0.038+	2.4	0.014+	4.1
B2-67		105/81	1:1	S1					0.039+	3.2	0.042+	2.1
B2-153		94/88	1:1	S3					0.046+	1.6	0.009+	4.0
B2-185		84/99	1:1	S3					0.0008+	5.3	0.019+	2.7
B2-314		128/52		S1					0.008+	3.2	0.003+	4.6
B3-53		5/188	1:35	C					0.019+	2.1	0.019+	3.0
B3-100	XII	10/182	1:11	C					0.011+	2.8	0.001+	6.4
B3-159		34/151	1:3	C					0.035+	1.5	0.026+	3.3
B3-305		85/99	1:1	S3					0.036+	2.3	0.019+	3.3
B3-327		100/90	1:1	S3					0.021+	3.6	0.035+	2.3
B3-352		138/51		S3					0.027+	2.7	0.051+	2.2
B3-425		132/44		S1					0.006+	3.9	0.064+	2.3
G3-129		29/156		C					0.002+	5.0	0.004+	4.5
G3-202	XII	115/65		S3					0.014+	2.2	0.026+	2.5
Y-393		43/128		S1					0.024+	2.0	0.030+	3.0
G3-54	IX	57/81		S1			0.034-	4.8	0.027-	2.8	0.010-	4.7
B1-241		22/169	1:5	S3					0.003-	5.7	0.003-	5.0
B2-58		58/124		C					0.004-	4.2	0.0005-	6.3
B2-76	V	90/95		C					0.0004-	5.9	0.0005-	6.9

Table 3-9 (continued)

					Reducing sugar content				Foliage earliness			
					Gransebieth		Windeby		Gransebieth		Windeby	
fAFLP marker	LG[a]	No -/+[b]	χ^2-test[c]	Ori.[d]	*P*[e]	R^{2f} (%)	*P*[e]	R^{2f} (%)	*P*[e]	R^{2f} (%)	*P*[e]	R^{2f} (%)
G3-206		44/138		S3					0.017-	2.7	0.017-	3.6
Y-459		106/65		S3					0.018-	2.0	0.036-	2.8
B1-182		26/115	1:3	C			0.044+	2.8	0.034+	2.7		
B1-330		68/73	1:1	S3	0.002+	5.1			0.036-	1.9		
B3-330		72/70	1:1	S3	0.023+	3.6			0.049-	1.6		
B1-77	XI	37/111	1:3	C	0.005+	5.4						
B1-102		51/95		S3	0.002+	7.3						
B1-455		90/52		S3	0.004+	6.7						
B3-141	V	70/76	1:1	S1	0.005-	5.3						
B3-211	I	63/81		C	0.007+	6.3						
G2-409		64/66	1:1	S1	0.006+	4.1						
B1-140		8/140	1:11	C			0.009+	4.0				
B1-261	IV	78/64	1:1	S1			0.005+	5.4				
B1-423		66/77	1:1	S3			0.004+	4.9				
B2-397		89/47		S3			0.004-	5.0				
G1-339		40/104	1:3	C			0.007+	5.1				
Y-111		13/120	1:11	C			0.009+	4.7				
B1-174	V	21/174	1:11	C					0.006+	3.5		
B1-322		48/138	1:3	C					0.007+	4.2		
B2-158		104/77		S1					0.001+	4.5		
G2-67		115/78		C					0.007-	2.7		
B2-68		21/164	1:5	S3							0.006+	4.0
G1-132		137/51		S3							0.009+	4.1
G2-80		76/110		C							0.002-	4.1
G2-120		35/157		C							0.004+	4.7
Y-83		83/92	1:1	S3							0.003-	4.5

[a] LG= linkage group, inferred from map positions of common fAFLP markers in the reference diploid mapping population K31.

[b] No. -/+, number of individuals analyzed showing absence/presence at the marker locus.

[c] 1:N, segregation type confirmed by goodness-of-fit test.

[d] Ori., origin of the marker with C representing common marker from both parents.

[e] *P* values followed by plus-minus the indicating the direction of the tagged QTL effect with '+' being increasing and '-' being decreasing RSC or FE.

[f] R^2= R square value in percent indicating the proportion of variance explained by the marker genotype.

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The only exception was marker B2-397, which decreased RSC. Only four markers were derived from the S1 parent, with two increasing RSC and two reducing RSC. The most significant markers are B3-117, B3-413, B1-330, B3-215, and G3-79. Individually, these markers each explained between 2.4 to 12.6% of the phenotypic variation among individuals for RSC.

fAFLP markers associated with FE

Compared to RSC, more fAFLP markers (32) were detected to be significantly associated with FE (Table 3-9). Effects of 23 markers were reproducibly detected ***(continue to page 79)***

Table 3-10 fAFLP markers showing associations with multiple phenotypic traits

					Tuber starch content				Reducing sugar content				Foliage earliness			
					Gransebieth		Windeby		Gransebieth		Windeby		Gransebieth		Windeby	
fAFLP marker	LG[a]	No -/+[b]	χ^2-test[c]	Ori.[d]	P^e	R^{2f} (%)	P^e	R^{2f} (%)	P^e	R^{2f} (%)	P^e	R^{2f} (%)	P^e	R^{2f} (%)	P^e	R^{2f} (%)
B3-409		138/51		S1	0.00017+	6.0	0.0018+	5.4			0.018-	3.9				
B3-134		143/40		S1	0.022+	1.6	0.017+	2.8			0.012-	5.0				
B1-214		36/109	1:3	C	0.015-	3.6	0.0051-	4.7	0.031+	3.8						
B1-166	I	100/94	1:1	S3	0.0069-	3.7			0.050+	2.2						
B1-77	XI	49/146	1:3	C	0.027-	2.9			0.005+	5.4						
B1-390		83/59		C	0.045-	2.2			0.023+	2.5						
B2-292		20/118	1:5	S1	0.013-	3.1			0.048+	2.8						
B2-62		79/103		S1	0.021+	2.9					0.039-	3.3				
G1-339		50/139	1:3	C	0.011+	3.6					0.007+	5.1				
G1-157		116/75		S1			0.025-	2.7			0.029+	3.8			0.019-	2.6
Y-452		51/120		S1			0.027-	2.8	0.037+	3.8	0.045+	3.1				
B1-112		77/117		C			0.035-	2.3	0.022+	2.5						
B1-149	III	9/183		C			0.028-	3.1	0.031+	2.8						
B1-364		112/70		C			0.040-	2.2	0.039+	3.2						
B3-121	IX	62/130		C			0.0035+	3.8	0.046-	2.3						
B1-174	V	21/174	1:11	C	0.045+	2.7	0.033+	1.5					0.006+	3.5		
G2-187		49/137	1:3	C	0.0037-	4.2									0.010-	2.9
B2-322		140/40		S1	0.019+	3.2							0.024+	4.7		
G3-199	IV	26/151	1:5	S1	0.019-	3.0									0.032-	1.8
B1-244		137/43		S3	0.018-	3.6									0.048+	1.9
Y-393		43/128		S1	0.021-	2.9							0.024+	2.0	0.030+	3.0
G1-200		140/49		S1			0.032-	2.3					0.016-	2.4		
B2-58		58/124		C			0.036-	2.8					0.004-	4.2	0.0005-	6.3
B3-100	XII	10/182		C			0.013+	2.2					0.011+	2.8	0.001+	6.4
B2-76	V	90/95		C			0.0011-	5.7					0.0004-	5.9	0.0005-	6.9
G1-205		103/86	1:1	S1			0.048-	2.0							0.047-	2.5
B1-242		107/76		S1			0.029-	2.6							0.015-	2.5

Table 3-10 (continued)

fAFLP marker	LG[a]	No -/+[b]		Ori.[c]	Tuber starch content				Reducing sugar content				Foliage earliness			
					Gransebieth		Windeby		Gransebieth		Windeby		Gransebieth		Windeby	
					P[d]	R^{2e} (%)	P[d]	R^{2e} (%)		R^{2e} (%)	P[d]	R^{2e} (%)	P[d]	R^{2e} (%)	P[d]	R^{2e} (%)
B1-330		94/47		S3					0.009+	3.6	0.006+	6.1	0.041-	1.6		
B3-215	V	84/60		S3					0.0004+	6.6	0.0001+	11.3	0.043+	1.9		
G3-79		82/58		S3					0.005+	7.8	0.029+	2.9	0.005+	4.6	0.001+	5.2
G3-54	IX	57/81		S1							0.034-	4.8	0.027-	2.8	0.010-	4.7
B1-182		26/115	1:3	C							0.044+	2.8	0.034+	2.7		
B3-330		72/70	1:1	S3					0.023+	3.6			0.049-	1.6		

[a] LG= linkage group, inferred from map positions of common fAFLP markers in the reference diploid mapping population K31.

[b] No. -/+, number of individuals analysed showing absence/presence at the marker locus.

[c] 1:N, segregation type confirmed by goodness-of-fit test.

[d] Ori., origin of the marker with C representing common marker from both parents.

[e] *P* values followed by plus-minus the indicating the direction of the tagged QTL effect with '+' being increasing and '-' being decreasing TSC or RSC or FE.

[f] R^2= R square value in percent indicating the proportion of variance explained by the marker genotype.

(continued from page 76)
over two locations, 17 of which resulted in delayed foliage maturity (positive effect noted with '+') while 6 lead to earlier maturity. The most significant ones were fragments B2-314, G3-129, B1-241, B2-58, B2-76, with effects accounting for phenotypic variation of 3.2 % - 6.9 % individually (Table 3-9, on page 75-76). Among these 23 markers, two effects with opposite direction were observed for markers of different parental origin. S1 alleles at the marker locus of G3-54 and S3 alleles at the marker loci of B1-241, G3-206, Y-459 were responsible for earlier foliage maturity, both S1 and S3 alleles at other marker loci resulted in delayed foliage maturity.

3.3.3.3 Association of fAFLP fragments with multiple traits

Of all the fAFLP markers analysed, 33 have been identified to be associated with more than one trait as detected by the t-test, regression analysis and the U-test. These markers were extracted and are shown in Table 3-10 (on page 77-78). 15 markers that were associated with TSC, also showed associations with RSC in at least one location. The most significant and reproducible ones were markers B3-409 and B3-134, which originated from S1 parent. These two markers exhibited effects of increasing TSC and decreasing RSC and thus, could be of potential use in potato breeding programs for MAS. It is worth noting that all markers had an opposite effect on the two traits, with the only exception being G1-339. In other words, markers with positive effects of increasing TSC would lead to decreased RSC or vice-versa. The results were in fairly good agreement with the phenotypic correlation relationship between the two traits as outlined in Section 3.3.2.

12 fragments showed multiple associations with TSC and FE. The most significant ones are G2-187, B2-58 and B2-76. Most frequently, the fragments with increasing effects on TSC usually lead to delayed maturity and vice-versa. The only exception was marker Y-393 for which the presence of the fragment decreased TSC and delayed maturity.

7 markers showed multiple associations with RSC and FE. The qualitative orientations of effects were variable among these markers. 3 markers (G1-157, B1-330, B3-330) with increasing effects on RSC lead to earlier maturity while 3 (B3-215, G3-79, B1-182) lead to delayed maturity. In one case, at locus G3-54, the decreased RSC was associated with earlier maturity.

One marker, G1-157 of S1 was associated with all three traits at one location (Windeby).

Presence of the marker decreased TSC, increased RSC and lead to earlier maturity.

3.3.3.4 Association analysis on 50 selected genotypes in trial 1999

In the trial 1999, 50 genotypes selected from the Z3 population were evaluated for various traits (see Sect.2.14). In addition to TSC and FE (RSC was not evaluated), other traits namely starch yield (SY) and tuber yield (TY) were also tested. The phenotypic data of these traits were, therefore, subjecteded to association analysis using the non-parametric U-test as described previously. The significantly associated fAFLP markers are shown in Table 3-11 (on page 81). A total of 42 fAFLP markers showed associations with starch yield, of which 18 and 21 were detected at either Gransebieth or at Windeby, and 3 were consistently detected at both locations. Of all these, 10 and 28 markers were also detected, at the same locations, to be associated with TSC and TY, respectively. This suggested that the effects of these markers on TSC and TY contributed to their effects on TY. 10 markers, which showed significant effects on either TSC or TY at the 1 % level, did not detect as having significant effects on SY.

Of the markers showing association with the traits in trial 1999, markers highlighted in bold (G1-75, B3-397, B2-428, B3-305, B3-51, G3-111G1-169, B1-215, B3-215, G3-54, and B3-352) were also detected, at least at the 5% level, as having association with the same traits in trial 1998. It is worth noting that for markers showing association with multiple traits, the directions of effects were uniformly in accordance to the phenotypic correlations (Table 3-11, page 81). In other words, the increase in either TSC or TY resulted in the increase of SY while leading to delayed maturity and vice versa.

3.3.4 Analysis of the segregation ratios in the tetraploid population Z3

Several segregation ratios were expected depending on the presence or absence of a fAFLP marker fragment in the parents. When a marker is present in only one parent, segregation ratios of 1:1 or 1:5 (absence versus presence) are expected for simplex and duplex markers, respectively. When a marker is present in both parents, segregation of 1:3, 1:11 and 1:35 (absence versus presence) are expected for simplex-simplex, simplex-duplex and duplex-duplex, respectively, without considering double reduction. Chi-square tests were performed to test for the goodness of fit to the expected segregation ratios. Markers fitting one of the expected segregation ratios were noted in Table 3-8, 3-9, and 3-10 (on page 73, 75-76, 77-78).

Table 3-11 fAFLP markers associated with phenotypic traits as detected by the U-test based on 50 selected genotypes in trial 1999

			SY[c]		TSC[c]		TY[c]		FE[c]	
	Pre/abs[b]	Ori./LG	Gra	Win	Gra	Win	Gra	Win	Gra	Win
B3-62	25/23	S3	0.012+	0.020+	0.0013+	0.008+				
G1-133	25/23	S3	0.024+	0.027+	0.033+	0.049+				
B1-144	36/12	C	0.028+	0.0005+				0.0002+		
G1-75[a]	30/19	S1	0.0013+			0.036+	0.0039+			
B3-269	16/25	S3/VIII	0.0028+				0.013+			
Y-60	30/15	S1	0.013+		0.021+					
G2-237	15/33	S1	0.038+		0.0081+					
G3-231	32/15	C	0.0078-		0.0073-					
G2-238	13/31	S3	0.019-		0.0048-					
G2-108	33/17	S3	0.032-		0.028-					
G2-301	21/29	S1	0.027+			0.013+				
B3-397	13/34	S1	0.029+			0.016+				
G1-106	34/15	S1/XII	0.040+			0.039+				
B1-464	19/28	C	0.039+					0.0067+		
G3-236	21/26	S1	0.010+				0.034+			
B3-305	25/20	S3/IV	0.011+				0.032+			0.046+
B3-223	40/7	C	0.014+				0.040+			
B2-428	30/18	C	0.029+				0.023+			
G1-105	27/18	S1	0.031+				0.034+			
B2-274	43/5	C	0.035+				0.0076+			
B2-324	15/33	S3	0.012-				0.022-			
B3-51	32/16	C		0.0025+	0.0016+	0.039+		0.013+	0.016+	0.013+
G2-120	40/9	C		0.0057+				0.019+	0.029+	0.037+
B2-185	22/26	S3		0.027+	0.046+			0.048+	0.0057+	
G3-111	33/14	C		0.0042+				0.026+		
B1-267	41/8	C		0.019+				0.0020+		
B3-104	43/5	C		0.023+				0.0059+		0.032+
B1-78	34/15	C		0.010+				0.009+		
B2-216	31/15	C		0.010+				0.013+		
G1-115	19/23	S1		0.013+				0.019+		
G2-164	18/25	S1		0.041+				0.0034+		
Y-393	38/8	S1		0.032+				0.012+		
B2-226	28/18	C		0.048+				0.049+		
G2-444	14/23	C/XII		0.031+	0.017+					
G1-250	37/11	C		0.041-	0.045-	0.011-			0.019-	0.0038-
B1-330	24/24	S3		0.0015-				0.0049-		
G3-387	20/22	S3		0.0048-				0.017-		
Y-97	34/12	S1		0.0064-				0.012-		0.043-
B1-141	28/21	S3		0.028-				0.036-		
B1-179	28/21	C		0.024-				0.034-		
B1-387	21/27	S3		0.045-				0.014-		
G1-130	37/11	C		0.043-		0.017-				
G1-169	8/37	S3/XII			0.0028-	0.032-				
G3-171	34/10	C			0.0055+	0.017+				
B1-101	38/10	C			0.031+	0.032+				
G1-157	22/27	S1			0.0082-					
B3-98	41/6	C			0.015+			0.031+		0.023+
G2-199	25/24	S3/VIII			0.018+			0.014+		
Y-247	37/9	C			0.021+			0.016+		
B3-111	15/31	S1			0.046+				0.014+	0.037+
B3-122	32/15	C/IX			0.039+				0.031+	
G3-338	26/18	C				0.0083+				
Y-120	39/7	C				0.0019+			0.017+	
G1-187	15/27	S1				0.049+			0.0086+	
B2-96	44/5	C				0.0082-				
B1-66	29/19	S1					0.007-			
B2-390	16/31	S3						0.0054+		
B1-215	35/10	S3						0.028+		0.041+
B1-133	40/9	C						0.034+		
B3-215	23/24	S3						0.047+	0.035+	0.017+
G3-358	16/29	S3						0.0018-		
B3-279	38/9	S3						0.0087-		
G3-54	32/14	C						0.011-	0.044-	
G3-206	38/8	S3						0.038-		0.045-
B3-352	16/31	S3							0.0091+	0.028+
B3-105	37/10	C							0.032+	0.0088+
B1-483	20/28	S3							0.0078-	0.0053-

[a] Markers highlighted in bold indicate that effects on the same traits were detected at least once at the 5% level in trial 1998
[b] Number of individuals with the marker presence versus absence,
[c] SY=starch yield, TSC=tuber starch content, TY=tuber yield, FE=foliage earliness, Gra=Gransebieth, Win=Windeby

3.3.5 Assignment of map positions to QTL-linked fAFLP markers

For fAFLP markers to be useful in a QTL study, they are ideally required to be assembled into maps showing their chromosomal locations. For this purpose, a strategy was adopted which is based on the assumption that fragments of identical size occupy the same map position in different mapping populations (van Eck *et al.* 1995). The reference mapping population K31 and LH was genotyped using the fAFLP technique with the same restriction enzymes and the same primer combinations as used for the Z3 population. Polymorphic fragments generated with the same primer pairs and having the same size as fAFLP markers of interest in the tetraploid Z3 population were selected for linkage analysis. The genotypic data, alongside the previously generated framework of RFLP data were analysed for linkages using the MAPRF© program (see Sect. 2.11). Of the fAFLP fragments that had shown associations with phenotypic traits (at 5 % significance level), 50 common fragments were identified which segregated in the reference diploid mapping population K31, and could be mapped, therefore, to potato linkage groups (Figure 3-10, on page 83). Of these, 9 fAFLP fragments (G3-393, B1-166, B3-260, G2-71, B1-316, B3-121, B3-130, B1-77, B1-103) which showed associations with TSC in Z3, were mapped within or closely linked to intervals of QTL for TSC as detected in K31 on LG I, II, III, IV, IX, X, XI and XII. 20 fAFLP fragments, which were associated with RSC in Z3, were positioned on LG I, III, IV, VI, VII, IX, XI and XII. 16 fAFLP markers associated with FE were localised on LG II, III, IV, V, VI, VII, VIII, IX, XI and XII. These fAFLP markers were scattered throughout the 12 linkage groups but were, to some extent, clustered, as seen on LG I, II, IV, V, VI, VII, IX, XI and XII. In many cases, clustered markers tagged QTL for the same traits, such as marker clusters for TSC on LG I, II, V, XII, and clusters for RSC on LG I, IV, V, XI. The most remarkable cluster of the fragments is on chromosome V where 9 fAFLP marker loci were clustered in a region of less than 30 cM. Most of these markers (6) showed associations with FE (Figure 3-10).

3.3.6 QTL analysis through candidate gene strategy

Five functional genes (*Dbe*, *Sss*I, *Stp*23, *Sut*2 and *Sus*4) selected on the basis of known overlapping positions with QTL for TSC as identified in population K31 (Schäfer-Pregl *et al.* 1998) were selected and tested for associations with putative QTL in population Z3. Genotypic CAPS data, which were generated from individuals of Z3 at the 5 gene loci, was analysed for associations with TSC, RSC, and FE using the appropriate ***(continue to page 84)***

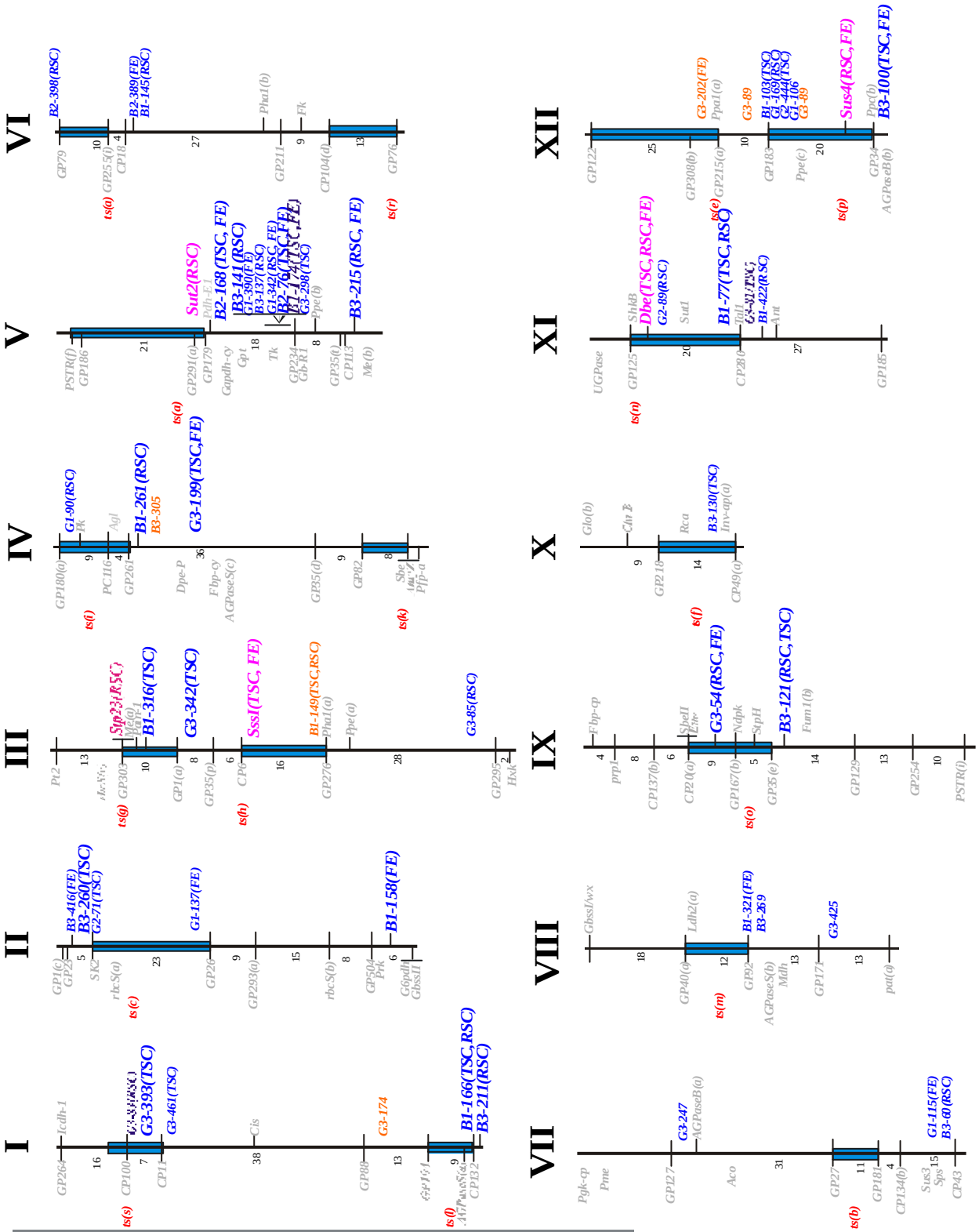

Figure 3-10
Map positions of fAFLP markers associated with phenotypic traits.
The skeleton map with markers shown in gray is adapted from Figure 3-3. fAFLP markers which are in blue and yellow were mapped in K31 and LH, respectively, whereas functional gene markers used for the QTL analysis are shown in red. The precisely positioned fAFLP markers are attached to the vertical map with small bars. Font size of the markers indicates the significance and consistency of the effects. Markers in large size are included in Table 3-8, 3-9, and 3-10, whereas small-sized markers indicate that they are detected only once at the 5% level

(continued from page 82)

tests as described in Sect. 3.3.3. Of the 5 genes tested, *Dbe-e* and *Sss*I, located on chromosomes XI and III, respectively, showed association with TSC in population Z3 at the 1% level (Table 3-12). The effects were both from an S3 allele, the presence of the allele *Dbe*-e at locus *Dbe* resulted in increased TSC, while the allele at the *Sss*I locus decreased TSC. The proportion of the phenotypic variance explained by the two loci was 3.5 % and 4.3 %, respectively. Besides the most significant allele *Dbe*-e, two other *Dbe* alleles from S3 were also detected as having significant effects (5 % level) on TSC at Gransebieth. At other gene loci no effects on TSC were detected in the tetraploid Z3 population. However, these loci showed associations with RSC. For instance, *Stp*23 and *Sut*2, located on chromosomes III and V, respectively, were consistently detected to be associated with RSC at two locations at the 1 % level (Table 3-12). The alleles detected were from parent S3 at both loci and accounted for 3.7 % - 7 % of the phenotypic variance with increasing effects on RSC. No significant effects on FE were detected at these gene loci.

In addition, multiple alleles were identified at loci *Dbe*, *Sus*4 and *Sss*I which made possible the subdivision of the population at these loci into four or more genotypic classes. Comparison of the mean of median values of these genotypic classes was achieved by applying the Kruskall-Wallis H-test. Only *Sss*I and *Dbe* showed significant effects ($P<0.05$) on starch and RSC respectively at Windeby (data not shown).

Table 3-12 Functional gene markers showing associations with multiple phenotypic traits

					Tuber starch content				Reducing sugar content			
					Gransebieth		Windeby		Gransebieth		Windeby	
fAFLP marker	LG[a]	No -/+[b]	χ^2-test[c]	Ori.[d]	P^e	R^{2f} (%)	P^e	R^{2f} (%)	P^e	R^{2f} (%)	P^e	R^{2f} (%)
*Stp*23	III	22/102	1:5	S3					0.008+	4.7	0.002+	7.0
*LeSUT*2	V	63/89		S3					0.017+	3.9	0.012+	3.7
Dbe-a	XI	82/68	1:1	S1							0.014-	4.7
Dbe-b	XI	101/97	1:1	S1								
Dbe-c	XI	129/58		S3	0.02+	2.5						
Dbe-d	XI	133/52		S3	0.04+	2.0						
Dbe-e	XI	143/52		S3	0.008+	3.5						
*Sus4**	XII	78/66	1:1	S1					0.06-	2.3	0.038-	2.6
*SssI**	III	93/98	1:1	S3			0.004-	4.3				

[a,b,c,d,e,f] see Table 3-10 (on page 77-78) for references

* two alleles of genes *Sus4* and *SssI* were identified, only the ones showing significant associations with RSC and TSC in the t-test were included in the table.

3.3.7 Cloning of fAFLP fragments

For breeding-oriented QTL analysis, it is highly desirable that significantly associated markers are amenable to marker-assisted selection. As a first step towards this purpose, fAFLP markers associated with a trait of interest need to be cloned. Since recovery of fAFLP DNA fragments directly from the automated fluorescent genotyping system is not technically accessible to date, this was bypassed by the use of the radioactive AFLP approach. Based on the results of association analysis, the five most significant and reproducible fAFLP fragments B3-117, 121, 215, 409, and 425 (see Tables 3-8, 3-9, 3-10, on page 73, 75-76, 77-78) generated with primer combination E-act/M-cta (B3) were selected for cloning. 10 individuals of Z3, for which the target fragments were well separated from the surrounding bands based on fAFLP fingerprinting, were tentatively selected for cloning. The same preamplification products of the two parents and ten selected individuals previously used for fAFLP were used directly for selective amplification. AFLP procedures were followed as described by Meksem *et al*. (1995). The AFLP size marker 30-330 bp with a 10 bp-ladder between neighbouring fragments was used to identify the fragments of interest. Of the five fragments examined, two fragments B3-409 and B3-215, at the expected position on an autoradiogram, gave rise to an identical segregation pattern to that of the fAFLP fragments. The other three fragments, which appeared faint on the autoradiogram, were not pursued further. The two identified bands were excised from the polyacrylamide gel, eluted and re-amplified with the corresponding non-radiolabelled primer pair to recover the DNA. This was successfully achieved for fragment B3-215 but failed for B3-409. The re-amplified PCR product of B3-215 fragment was subsequently cloned into the pGEM®-T vector. Putative recombinant colonies were either confirmed by colony PCR which was performed directly using bacterial colonies on agar plates as templates or alternatively, by digestion of the extracted plasmid DNA with restriction enzymes. Plasmid DNA from 10 confirmed colonies was prepared and subjected to sequencing. All 10 sequences showed the selective primer sequence of *Eco*RI at one end and the *Mse*I primer sequence at the other. The analysis of the 10 sequences revealed three different sequence classes. One was 218 bp in length; two were 219 bp sequences; the third one was 217 bp in size with 7 members, which showed high similarity (98-99%) and differed from one another only by single nucleotides (Figure 3-11). The majority class had a 2 bp discrepancy from the expected 215 bp. This discrepancy may have resulted from the automated sizing by the GeneScan® system by which size assignment may have 1-2 bp variations depending on the size categories defined. Taking into account

Primer E-cta

5′-**GATGAGTCCTGAGTAActa**GGTTCG(G/A)CAAACTTTTGTTTTCTTTTGCCTA
CTCCTCGGACACAACAACCAATATTTATTTCACTC(C/T)AAAAGA(G/T)TAC
AAGTGAAATACTACAAGAGAGAAAGAAGATCAAATGCCTTTGAAGAT(G/A)A
(G/A)AAGGCAAG(T/C)GAGAGGTGTGT(T/C)ACAAATGAATTAGGAACTAC
CTATTTAT(A/G/T)GG**agtGAATTGGTACGCAGTC**-3′

Primer M-act

Figure 3-11 The combined consensus sequence from the 7 sequences of the cloned fAFLP fragment B3-215. Nucleotide variations among the 7 sequences were indicated in parentheses. Primer sequences were underlined and lower case residues were selective nucleotides.

this fact, the 7 sequences were assumed to be the correct ones. A BLAST search for homologous sequences in the data bases indicated that this sequence showed 88% homology at the nucleotide level to a downstream sequence segment of the ORF of the gene coding for chlorophyll a/b binding protein (*Lhcb*1, accession No. AB012636) in *Nicotiana sylvestris*. For confirmation and PCR-marker conversion of this cloned fragment, various approaches were tested. First, since the complete sequence was known, a primer pair was designed by adding four more sequence-specific nucleotides at the 3' end of each selective primer. The idea was that using more selective primer to amplify the pre-amplification product used for fAFLP generation would result in a single or few fragments resolvable on agarose gel with segregation pattern identical to the fAFLP B3-215 marker. Although a single fragment with the expected size was obtained under stringent amplification condition, no polymorphisms were exhibited between Z3 individuals. This may be due to the fact that the selectivity of the primers was reduced or even lost after addition of the four sequence specific nucleotides at the 3' end of the primers. An alternative option for confirmation could be DNA blotting, by which gel-electrophoresis-resolved AFLP fragments are transferred on to membrane followed by hybridization with a probe from the cloned AFLP fragment. This approach was, however, not applied in considering that the final goal was to convert the fAFLP marker into PCR-based marker, which, when achieved, would also lead to the confirmation of the segregation pattern. For marker conversion, more sequence information is needed. The high sequence similarity of the cloned AFLP fragment to the gene *Lhcb*1 of *Nicotiana sylvestris* was used to

achieve the goal by amplification of the homologous gene segment in potato population Z3. A primer pair was designed with one primer being derived from the most conserved region of *Lhcb*1 gene, the other from the sequence of the cloned fragment. The two primers encompassed the *Mse*I restriction site of the AFLP fragment. A single fragment of 500bp in size was amplified from both parents S1 and S3 using genomic DNA as template. This PCR product was digested with restriction enzyme *Mse*I to test if the *Mse*I site was the cause of yielding the fAFLP fragment. However, the cleavage of the PCR product with *Mse*I did not detect CAPS between the two parents. Sequencing of the PCR fragment from S1 revealed high sequence homology (93 %) to the cloned fragment B3-215 and also an *Mse*I site as expected (Figure 3-12). The point mutations between S1 and S3 allowed designing allele-specific primers, however, PCR amplification failed to distinguish them. This is not unexpected since the allele-specific primers were base on the difference of only one allele from S1 whereas the other three remained unknown. Consequently, the fAFLP fragments B3-215, while successfully cloned, is still to be confirmed.

```
  1  GAGAACCTTG  CTGACCACCT  TGCTGAAGTT  TGTGTCTTGC  GATTAGCAAT
 51  CGTAGGGATT  TTCTTATAAC  AGGATCACAT  GATCGTTCCA  TACGACGTTG
101  GGATCGTACT  GATGATGAAG  CTCTTTTTAT  TGAGGTGTTA  TATTTTGCAT
151  CAGTTTTAGA  CAGTTAAAAA  GAATAATTTA  TAAAGTTGCT  ATTTGGAATT
201  GCAGGAAGAG  AAAGAAAAGA  GATTGGAAGA  GATGTTTGAA  TAAGATATCG
251  ATAATGTATT  TGAGAATAAG  TATGTGTTGT  TGAAGCCGAA  TATATAGAGA
301  GTAATGGAAT  CGCAGCTGCT  ATATCTAAAG  GTAGCTAATA  AATAGTAAAT
351  GAGATAACAA  TAAAAAGAAC  ACCATGAATT  AACGAGGTTC  GGCAAAATTT
401  TGATTTCTTT  TGCCTATTCC  TCGGACACAA  CCAACCAATA  TTTTTACCAT
451  TCCAAAAGAG  TACTAGTAAA  ATACTACAAG  AGAGAAAGAA  GATCAAATGC
501  CTTAGAAGAT  GAGAAGGCAA  GTGAGAGGTG  TGTTAC
```

Figure 3-12 Extended nucleotide sequence of the cloned fAFLP fragment B3-215. Sequence was derived from parent S1, nucleotide residues in italics indicate the sequence of the cloned fAFLP fragment, others are extended sequence starting from *Mse*I site (highlighted in bold). Primers sequences are underlined.

4. DISCUSSION

4.1 Mapping functional genes involved in carbohydrate metabolism and transport

4.1.1 STS as an approach for functional gene mapping

In the past decade, many advances have been made in the construction of molecular maps for crop plants using various molecular marker tools such as RFLP, RAPD, SSR and AFLP. These types of molecular linkage maps play an important role in the genetic analysis of agronomic traits including map-based gene cloning and QTL analysis. However, all these types of markers are largely anonymous and of unknown function, limiting further exploration of the genetic basis underlying the marker-tagged agronomic traits studied. Thus, the construction of linkage maps with markers tagging known functional genes is highly desirable and, when achieved, could take genetic mapping to a further level. Typically, Southern hybridization of a segregating population with a cloned gene as probe is the common way to localize the gene on molecular linkage map and 22 genes have been mapped through this approach (Gebhardt *et al*. 1994). Although RFLP is notably reliable for mapping, the application is limited by the availability of a clone for the gene of interest and additionally, the marker created could hardly be incorporated into MAS for screening plants on a large-scale because the technique is laborious and time-consuming.

In the present study, public databases were systematically searched for sequences of plant genes with a functional role in carbohydrate metabolism and transport. Sequence information was used to develop PCR-based marker assays that allowed localization of corresponding potato genes on existing RFLP linkage maps. This approach takes advantage of existing sequence resources in the databases for gene-specific markers, thus enabling the efficient and successful construction of molecular maps for functional genes with minimal additional requirements. The highly polymorphic mapping populations K31 and LH offer a good foundation for the positioning of functional genes on potato linkage maps through an STS approach. All of the genes in this investigation were mapped through this approach using one or other of the two populations except gene *Tk* for which no polymorphism was detected. Alternatively, *Tk* was mapped by a RFLP assay using a PCR product of *Tk* as a probe. Using

the STS approach in combination with RFLP, an initial potato molecular function map based on RFLP mapping of cloned genes (Gebhardt *et al*. 1994) was expanded about four-fold.

To map functional genes efficiently and successfully through the STS approach, selection of the appropriate gene regions for primer design is critical since the majority of gene sequences available in databases are cDNA sequences. It was found to be important for primer design to avoid the exon-intron boundaries and to position the two primers at a distance of 500-800bp apart from each other on the cDNA sequence so that PCR products of sufficient length encompassing introns could be amplified for identifying polymorphisms. Two methods gave an indication of such exon-intron junctions. First, some sequence motifs such as CAGG or AAGG may be a hint of the junction sites (Lefebvre and Gellatly 1997). Thus primer design should avoid these sequence motifs. Second, if the genomic gene sequence of another related species is available, the comparison of the cDNA sequence with the genomic sequence could lead to the identification of putative introns.

4.1.2 Validity of the STS approach for mapping functional genes

In the present study, the first technical choice for function mapping was the STS approach because of (i) the availability of large number of nucleotide sequence information of genes in the GenBank and EMBL databases; (ii) the ability to specifically detect by PCR genes known in different genomes using degenerate primers complementary to exon sequences (Larrick *et al*. 1989, Holland 1993, Gould *et al*. 1989, McPherson *et al*. 1991 and Van Campenhout *et al*. 2000); (iii) the possibility of directly sequencing PCR products, which is the best way to validate gene homology and (iv) the feasibility of routine application of the markers in MAS. The reliability of the STS approach was confirmed by sequencing the PCR products and comparison of their map positions. First, the specificity of the PCR amplification was verified by sequencing three PCR products generated with primers from potato sequence and all six PCR products obtained with degenerate primers from other species than potato. The former cases showed 95 % -100 % similarity and the latter cases showed 80 % - 95 % similarity with the reference sequences. Second, seven genes were mapped by both the STS approach and by RFLP using different mapping populations and all were localized in similar positions on potato linkage map. This suggests that the employment of an STS approach for function mapping is reliable.

4.2 The potato function map

4.2.1 PCR-based markers for genetic analysis and marker assisted selection

The functional genes mapped by the STS approach in the present study, together with other previously mapped genes, creates a framework of 66 gene markers opening up new possibilities for various genetic studies in potato. On one hand, since the genes mapped are all genes with known function and mostly involved in carbohydrate metabolism and transport, thus candidate gene strategy becomes accessible to analyzing QTL for tuber starch and sugar content in different populations. This could be achieved by genotyping with the candidate gene markers for their segregation in populations considered and testing their significance of effects on the traits of interest. This approach can eliminate the generation of the genome-wide markers by anonymous marker technology, and has been proven fairly efficient in QTL studies as described in Section 3.3.6. On the other hand, since the gene markers as defined in this study are PCR based, these markers, together with previously developed sequence based anchor markers (Oberhagemann *et al*. 1999) are a valuable tool for genetic analysis and marker-assisted selection in potato. They (i) cover a considerable proportion of the potato genome, (ii) are available as locus specific sequence information and (iii) can be analyzed by PCR with small amounts of genomic DNA as template; (iv) may be further optimised for large-scale screening through the use of high throughput techniques like molecular beacons (Tyagi and Kramer 1995; Tyagi *et al*., 1998), thereby eliminating gel electrophoresis. Marker assays will have to be verified and adapted in order to be informative, however, when applied to germplasm other than the material used for gene mapping in this study.

4.2.2 Molecular function maps for comparative studies of plant genomes

Most of the genes for which map positions are reported in this paper are housekeeping genes functioning in general plant metabolism. Even distantly related plant species have in common these genes with similar functions and a high degree of sequence conservation. In different plant species, molecular maps based on common genes may be used not only for comparative studies of plant genome evolution, but also for functional comparisons. Few molecular maps are, however, currently available in plants for specific sets of genes of known biochemical and physiological effect. Similar studies have been carried out in maize and sugar beet (Causse *et al*. 1995, Schneider *et al*. 1999). The sequencing of whole plant genomes as done

for *Arabidopsis* (Bevan *et al*. 1998, Lin *et al*. 1999) or the construction of linkage maps based on expressed sequence tags as done in rice (Kurata *et al*. 1994) and maize (Chao *et al*. 1994) makes available information on genomic positions of functionally and structurally similar genes which can also be used for genomic comparisons. For the potato and tomato genomes, extensive colinearity has been demonstrated (Bonierbale *et al*. 1988). In tomato, a genetic map including isozyme loci and more than 300 randomly selected EST markers has been constructed (Tanksley *et al*. 1992). Based on their DNA sequence, putative functions have been assigned to tomato EST markers (Ganal *et al*. 1998). The availability of a potato function map made possible the evaluation of synteny between potato genes and tomato EST markers, to which the same functions have been assigned based on sequence similarity (Ganal *et al*. 1998, Tanksley *et al*. 1992). CAPS markers for potato genes *Icdh-1* and *Aco* mapped to positions on linkage groups I and VII, respectively, which are in good agreement with isozyme loci *Idh-1* and *Aco-2* on the tomato molecular map. Potato genes *UGPase* on linkage group XI, Pgk_{cp} and *Pha2*, both on linkage group VII, occupy similar map positions as tomato markers *CT182*, *CT114* and *CD54*, respectively, which code for the same enzymes (Ganal *et al*. 1998, Tanksley *et al*. 1992). In four cases, the potato gene marker identified a single locus, whereas two or more loci were identified by the corresponding tomato EST marker. The four loci were: *AmyZ* (LG IV), *Stp1*, *Tk* (both LG V) and *Ndpk* (LG IX), which correspond to tomato marker loci *CT224B*, *CD31A*, *CD38B* and *CT225A*, respectively, to which the same function has been assigned based on sequence similarity (Ganal *et al*. 1998, Tanksley et al. 1992). In one instance, the CAPS marker for the potato *Ppe* gene identified three loci in potato on linkage groups III, V and XII, whereas tomato marker *CT248* for *Ppe* detected only the locus on linkage group III. No correspondence between map positions was found for the potato *Me* gene for NAD^+-dependent malic enzyme (LG III and V) and tomato marker *CT201* which is similar to $NADP^+$-dependent malic enzyme. The DNA sequences of the two markers share only 56 % similarity. The map positions of potato genes for fructose-1,6-bisphosphatase (Fbp_{cp} on LG IX and Fbp_{cy} on LG IV) were both different from the position of tomato marker *CD5* (LG X) which has sequence similarity with the same enzyme. The potato homologue of a tomato glycolate oxidase gene (*Glo*), although 96.6 % identical with the tomato sequence from which it was derived (Table 3-2), detected two loci on potato linkage groups VII and X, which were in disagreement with the position on tomato linkage group II of tomato marker *CD79* which is identical to the tomato *Glo* gene sequence used for primer design (M. Ganal, personal communication).

In potato and tomato, orthologous genes with conserved sequences and the same function assignment are expected to occupy similar map positions. This was, in fact, observed for most of the genes examined, when taking also into account the different numbers of loci detected in several cases in the two species. The difference in the number of loci identified with the same gene marker might be due to (i) the fact that not all loci of a gene are identified due to the limited DNA variation in a given mapping population or limited power of the techniques used for mapping. For this reason, the number of loci detected may be considered as a minimum estimate. Consequently a locus may be detected in both species, another locus may be identified in one species but not in the other or vice versa; (ii) the nature of the marker assays used for mapping. The RFLP assay identifies, depending on the hybridization stringency used, loci with 80 % or less sequence identity with the probe, whereas a PCR-based assay requires higher identity between primer and target sequence and is, therefore, more gene specific. Alternatively, the potato and tomato genomes may have indeed different numbers of copies of certain genes (Gebhardt *et al*. 1991). Three gene markers, however, did not occupy syntenic positions on the potato / tomato maps. First, this might be due to erroneous functional assignment when sequence similarity is not extremely high as in the case of malic enzyme (*Me*) where the potato gene shared only 56 % similarity with the tomato EST. Second, in the case of gene families with members that are not linked, different loci might be polymorphic in the two species compared. Third, members of gene families with high interspecific sequence conservation compared to other members of the same family may not always be truly orthologous. Differential selection pressure acting on members of a gene family after speciation may have resulted in higher sequence divergence between orthologous members than between paralogous members. This might explain why a glycolate oxidase (*Glo*) gene marker, although highly conserved between potato and tomato, was located on different linkage groups in the two genomes.

4.2.3 Candidate genes for TSC (TSC)

Quantitative trait loci (QTL) for TSC and tuber yield have been mapped in the populations K31 and LH (Schäfer-Pregl *et al*. 1998). The marker intervals with approximate positions of tuber starch QTL as previously identified are indicated in Figure 3-3. The same populations were used for PCR-based mapping of genes involved in carbohydrate metabolism and transport. Comparison of the QTL map with the molecular function map reveals a number of correlations between map positions of QTL for TSC and functionally related loci. This was

expected, due to the map coverage by both the QTL and the functionally related loci. Overlapping chromosomal positions are observed, either when the gene(s) responsible for a QTL effect are linked but functionally unrelated to a marker gene, or when the marker gene itself is responsible for the QTL effect. Evaluation of the latter possibility has to be knowledge-based, considering the physiological, biochemical and molecular role of candidate genes.

The most direct candidates controlling TSC are genes encoding starch-metabolizing enzymes. Sixteen genes of this class were mapped yielding eighteen loci on the potato molecular function map (Gebhardt *et al*. 1994 and this study). ADP-glucose pyrophosphorylase is a key enzyme of starch biosynthesis (reviewed in Frommer and Sonnewald 1995). Markers for the two subunits S and B of potato AGPase identified five loci one of which, *AGPaseS(a)* on linkage group I, was positioned in the same 9 cM marker interval as the QTL *ts(l)* (Figure 3-3) having a small effect on TSC (Schäfer-Pregl *et al*. 1998). One of four mapped starch synthase genes, *SssI* on linkage group III, is linked to QTL *ts(h)*. Genes *SbeI* for starch branching enzyme I and *Dbe* for debranching enzyme are linked to QTL *ts(k)* and *ts(n)* on linkage groups IV and XI, respectively. Five genes coding for starch degrading enzymes were mapped: *Stp23* on linkage group III, *AmyZ* and *Agl* both on linkage group IV, *Stp1* on linkage group V and *StpH* on linkage group IX. All five loci are linked to QTL for TSC (Figure 3-3). The clearest positional correlation was between *Stp23* and QTL *ts(g)* on linkage group III because both the QTL and the marker gene are most closely linked to the same anchor RFLP marker *GP303* (Figure 3-3 and Schäfer-Pregl *et al* 1998). This suggests that natural allelic variants of genes controlling starch synthesis and degradation may contribute to quantitative effects on TSC.

Accumulation of tuber starch also depends on availability of substrate for starch synthesis and, therefore, more indirectly on photosynthesis, sugar metabolism, transport and energy supply (reviewed in Frommer and Sonnewald 1995). Allelic variants of genes operating in these metabolic pathways may be responsible for variation of TSC. Known genes in these pathways were placed, therefore, onto the potato molecular function map. The list of the genes mapped is, by no means, complete. QTL for TSC were linked, among others, to loci for ribulose bisphosphate carboxylase, small subunit (*rbcS-c* and *ts(c)* on LG II, *rbcS-1* and *ts(g)* on LG III), ribulose bisphosphate carboxylase activase (*Rca* and *ts(f)* on LG X), sucrose synthase 4 (*Sus4* and *ts(p)* on LG XII), pyruvate kinase (*Pk* and *ts(i)* on LG IV), pyrophosphate fructose-6-phosphate 1-phosphotransferase, α subunit (*Pfp-a* and *ts(k)* on LG

IV), malic enzyme (*Me(a)* and *ts(g)* on LG III), plasma membrane H^+-ATPase 2 (*Pha2* and *ts(b)* on LG VII) and soluble inorganic pyrophosphatase (*Ppa1(a)* and *ts(e)* on LG XII), soluble acid invertase (*Pain-1* and *ts(g)* on LG III) and apoplastic invertase (*Inv-ap(a)* and *ts(f)* on LG X)

In many cases, the same QTL was linked to several candidate gene loci. For example, at least three loci, *rbcS-1, Stp23* and *Me(a),* overlap with QTL *ts(g)* on linkage group III and three others, *SbeI, AmyZ* and *Pfp-a*, overlap with QTL *ts(k)* on linkage group IV. But from this study it is unclear whether all are contributing equally or if only one is the primary contributor to the overlapping QTL or if other unknown genes at these regions are responsible for the effects. Several strategies can be adopted for further study to validate a putative candidate gene. First, QTL analysis may be carried out based on activity measurements of the candidate enzymes as performed in maize (Causse *et al*. 1995); second, transformation of a genotype with various alleles may be conducted for expression analysis and functional analysis of molecular variants. In this case, however, allele-trait association may go undetected if different alleles have a similar influence on the trait.

Three of the seventeen QTL for TSC (*ts(s), ts(a)* and *ts(r)*) shown in Figure 3-3 were not linked to any known candidate gene locus. QTL *ts(s)* on linkage group I was linked to acidic and basic β-1,3-glucanase genes (*GluA* and *GluB*) which, like chitinases (*ChtB* on LG X), are related to pathogenesis and defense (Kombrink and Somssich 1997) response to diseases rather than to starch accumulation in tubers. Therefore, they are not considered as candidate genes for this trait despite their linkage to QTL for TSC.

The present study focused mainly on the structural genes coding for various enzymes functioning in the carbohydrate metabolism and transport. It is clear that regulatory genes controlling transcription, translation and posttranslational modification of the starch metabolic enzymes play also a pivotal role in these processes. For example, the *p1* locus in maize which encodes a transcription factor regulating the synthesis of compounds in the flavonoid pathway was proved to be an important regulatory locus as a QTL for resistance of maize to corn earworm (McMullen *et al*. 1998). However, regulatory factors have not been reported for starch metabolism to date. While the physiology, biochemistry and molecular biology of carbohydrate metabolism and transport in plants have been extensively studied, the information on the total number of genes relevant to TSC is incomplete and will remain so for the foreseeable future. Nevertheless, there are a few lines of evidence suggesting that a

molecular function map for the genes currently known to control carbohydrate metabolism and transport is a good basis for explaining at the molecular level QTL for starch- and sugar-related agronomic traits in plants. Apart from the results of our study as outlined above, in maize, all mutations affecting the starch content of the kernel that have been identified at the molecular level affect enzymes of carbohydrate metabolism (Neuffer *et al.* 1997). QTL for sugar content of kernels and other traits of maize are linked, for example, to QTL for enzymatic activity of sucrose phosphate synthase (Causse *et al*. 1995). Moreover, a first QTL for sugar content of tomato fruits has been characterized on the molecular level and shown to be controlled by molecular variants of an apoplastic invertase gene (Fridman *et al*. 2000).

4.3 Single nucleotide polymorphism (SNP)

SNP is a new type of molecular marker that is very useful and robust in genetic association analysis. SNPs are detected by various techniques, for instance, by gel-based sequencing (Nickerson *et al*. 1997), high-density variation-detection DNA chips (VDAs) (Wang *et al*. 1998), and direct approach based on public databases (Picoult-Newberg *et al*. 1999). In the present study, an attempt was made to identify the potential SNPs existing in the gene *Stp*23 by sequencing PCR products of the gene derived from different genotypes. The template DNA used for sequencing was PCR product amplified with the same primers from both diploid and tetraploid potato lines. The objective was to investigate the feasibility of detecting potential SNPs by sequencing PCR products of the gene of interest and, to characterize the SNP distribution in functional genes. The results of SNP obtained, although they need to be confirmed, represent a promising beginning for further identification and application in potato genetic studies.

In this study, the identification of SNPs has been demonstrated (Sect. 3.2) by visually examining the electropherogram of sequence data from a PCR product. Overlapping peaks at a single nucleotide position indicates a potential SNP (Figure 3-6). The SNPs identified by this way reflected the heterozygocity of the genotype. When comparing the peak height of a nucleotide signal in the homozygous state with those in the heterozygous state (potential bi-allelic SNPs), a characteristic drop in peak height at heterozygous positions for two peaks is observed on the electropherogram and the two peaks result frequently in a base called 'N' (Figure 3-6). This feature can be used to detect heterozygous positions (SNPs) from sequence data. The basis of this feature may be the different dosage of the single nucleotide templates.

In effect, when sequence data were based on a PCR product which is the mixture of two alleles (in diploids), nucleotide dosage of the template can be reflected by the signal intensity or peak height on the electropherogram. Obviously at heterozygous positions, the template of each nucleotide is only half concentrated compared to those at homozygous positions. Thus, a drop of peak height at SNP positions is expected on the electropherogram. This feature was observed for all bi-allelic SNPs identified with only exception of the consecutive tri-allelic and bi-allelic SNPs found in tetraploid line Taiga (Figure 3-6).

The only drawback for the identification of SNPs through direct sequencing of PCR products is that it requires high quality sequence data to give a reliable discrimination between heterogeneity of alleles and signal noise. High quality sequence data is needed for confirmation of putative heterozygous / homozygous position.

Sequencing from cloned PCR products gives high sequence quality, however, additional work like cloning of PCR products into vectors and multiple clones are required. For this reason, sequencing directly from PCR products has more advantages in terms of efficiency, particularly for tetraploid lines.

Besides the identification of SNPs as heterozygous nucleotides within single lines, sequence assembly of different lines can detect mismatches for potential SNPs among different lines. Such SNPs reflect the frequency that a position within the assembly has heterogeneity in nucleotide composition. This depends on the number of sequences compared and the genetic diversity among them.

For large-scale identification of SNP by sequencing, efficiency could be enhanced by sequencing pooled DNA of different sources rather than numerous individuals as done in the present experiment. Additionally, the procedures of sequence analysis could be automated by use of computer programs *Phred* for base calling, *Phrap* for sequence assembly and *Consed* for sequence assembly editing (Nickerson *et al*. 1997).

In general, a potential SNP for a single copy gene may be considered as real when the fluorescence-based sequence was of high quality. However, care must be taken when interpreting the results. First, the base-calling error of sequencing exists, which can be overcome by repeated sequencing of the same sequence segment in different genotypes. Second, the possible paralogous of a gene family should be considered. In cases when the gene examined is a member of a multigene family, the candidate sites that appear

heterozygous in a sequence, may be the result of mismatches between paralogous members of the multigene family. This issue can be further addressed by assessing the genetic distance between members of the sequences.

For aspects of application, SNP data of genes are required, which are representative for germplasm potentially used in breeding, so that SNPs identified are likely to be informative in any breeding families, thus increasing the potential of applicability of SNP data. Therefore, a survey of SNP using a sufficient number of materials is needed. The sequences assembled for SNP discovery in gene *Stp*23 represented some commercial and elite breeding lines used in breeding program. They may not be sufficient to achieve a good coverage of commercial breeding materials. They may also represent only a limited genetic base of potato since the cultivated potato in Europe was derived from a limited number of *Solanum tuberosum* ssp. *andigena* (Bradshaw *et al*. 1994). Therefore sequence differences observed in the 14 lines represent only part of the SNPs potentially existing in the potato gene pool.

In the SNP analysis of gene *Stp*23, eight exonic SNPs were revealed, of which four derived from the T710 parent of population LH and one from the P3 parent of population K31. These SNPs resulted in amino acid changes, which may further modify the gene function and expression, and ultimately affect the phenotypic trait like TSC. Interestingly, a remarkable effect on TSC was observed at the locus *Stp*23 in both two populations K31 and LH (Schäfer-Pregl *et al.* 1998). This coincidence may imply that *Stp*23 could be a causal gene affecting the TSC and the non-synonymous changes of amino acid observed in this gene could be the cause. It can not be ruled out, however, that SNPs at other regions, for example in the promoter region, could also be the cause for the phenotypic variation of TSC. When the gene causing the effect is validated, the effect of the allelic variations of the genes could be actually the real genetic factors affecting the quantitative trait, thus circumventing the map-based cloning of the QTL.

The SNPs identified occurred in exons as well as in introns. The former ones were much less frequent than the latter ones. This feature was also observed in other organisms (Li and Sadler 1991). In addition, SNPs are frequently distributed in the total genome of all organisms examined so far. For instance, a frequency as high as 1 in every 202 bp has been observed in mouse genome (Lindblad-Toh *et al*. 2000). Because of this, SNPs have many advantages and much potential for application. First, it can facilitate the development of high-density genetic maps because of the high occurrence in the genome. Second, since the

detection of SNPs can be focused on structural genes which are more valuable than anonymous regions. Therefore, SNP, as a new marker system, can provide an alternative to the STS approach for function mapping. Third, this approach could identify a number of sequence variants in exons or promoter regions that may lead to the alteration of gene function or gene expression. This could be associated with phenotypic variation in the population. The allelic variations of the gene may actually be the genetic factors affecting the quantitative trait. Finally, as the sequence context of the SNPs is already known, it is possible to exploit some of the automated approaches available to genotype a population on a large scale.

4.4 QTL analysis

4.4.1 Strategy of QTL-tagging

In potato, QTL analysis is largely constrained to the experimental diploid populations to avoid the complexities of tetrasomic inheritance (Bonierbale *et al*. 1994; Leonards-Schippers *et al*. 1994; Van Eck *et al*. 1994, Freyre *et al*. 1994, Van den Berg *et al*. 1996a, b; Douches and Freyre 1994; Freyre and Douches 1994; Schäfer-Pregl *et al.* 1998). Studies performed on diploids provide informative insight into the genetic nature of the trait studied. Making inferences from the diploid to the tetraploid level is not straightforward, however, since genes affecting quantitative traits might have different effects at the diploid and tetraploid ploidy levels (Groose *et al*. 1988). Thus, it is highly desirable that breeding-oriented QTL analysis is performed on tetraploids.

In general, QTL analysis requires construction of a linkage map with markers evenly distributed over the genome using the population from which phenotypic data were collected. However, due to the complexity of tetraploid genome structure, linkage construction in tetraploid potato is difficult. First, a total of 96 linkage groups (four homologous groups of 12 chromosomes for each parent) potentially exist for a tetraploid population of potato. For linkage mapping, an extremely large number of markers, preferably codominant have to be generated to have a sufficient number of markers on each group. Second, bridging markers need to be generated to identify the homologous groups. Apparently, the efforts leading to the linkage construction is expensive and time consuming, which has been demonstrated by Meyer *et al.* (1998). In their experiment, 573 AFLP markers that segregate in three segregation types (1:1, 1:3, 1:5) were selected for linkage analysis from the fragments

generated with 39 AFLP primer combinations. These markers could be organized into 30 and 26 linkage groups for the maternal and paternal maps, respectively, representing 25 % coverage of the genome. Only 8 homologous groups were identified because of the limited number of bridging AFLP markers linking homologous groups.

In present study, without a prior construction of linkage map, QTL were tagged by fAFLPs using fluorescent sequencing system in combination with t-test regression analysis and U-test. This is the first study on QTL tagging in a tetraploid crop plant using fAFLP marker technology. This strategy was adopted because, first, fAFLP is a semi-automated genotyping technique which allowed to generate with high efficiency sufficient amount of molecular markers in a short period of time compared to other available marker technologies. Second, the dominant nature of fAFLP markers being scored as presence versus absence, meets the requirements for QTL tagging by parametric t-test or non-parametric U-test and the quantitative fAFLP data also allowed correlating fAFLP markers with phenotypic traits by regression analysis. The power of these statistic tools may not be equally high when applied to data of markers with different segregating types. For example, segregation ratio deviated from 1:1 may reduce the power of the t-test. However, these two methods, when combined, can complement to each other and could give a fairly good association test for markers with different segregation types. And finally, this strategy needs not to generate so many markers as required for linkage mapping in tetraploid. In effect, when sufficient markers are generated to cover 12 chromosomal linkage groups irrespective of the homologous groups, further increase in number of markers may enhance the precise detection of QTL but may not significantly lead to the identification of new QTL. In the present study, more than 200 polymorphic fAFLP markers were estimated to be generated with 7 primer combinations (see Table 3-5). These markers, although much less than sufficient to generate linkage groups or even to identify marker pairs linked in coupling-phase, may give a fairly good overall chromosomal coverage. Van Eck *et al.* (1995) generated a total of 264 markers with six AFLP primer combinations and demonstrated a good overall genome coverage of the diploid potato genome. The fAFLP markers generated in this experiment, therefore, have the potential to tag the QTL dispersed in the total genome of Z3.

This approach, while successful in QTL-tagging, suffers some inherent weakness. On one hand, despite the fact that a number of fAFLPs were inferred from common AFLP markers identified in diploid reference mapping population (see Sect. 3.3.5), most fAFLP markers of interest still remain unknown in terms of their chromosomal positions. Consequently, it

remains unclear (i) whether QTL markers which showed associations with the same trait were linked or independent, (ii) how many QTL were identified and, (iii) to what extent the QTL can be exploited for MAS in the population, which, in case when the number of QTL is known, can be estimated from the total proportion of phenotypic variance explained by all QTL. On the other hand, the dominant nature of AFLP prevented discrimination of allelic composition of the tagged loci in the parental lines, and thereby, analysis of configuration and the effect of different combinations on the trait of interest were not feasible. This is particularly important in polyploid genomes where a greater number of possible alleles at a locus exist.

Despite the drawbacks, the fAFLP data of population Z3 does provide valuable information regarding the genetic components at all tagged loci for each individual of the population Z3. The fAFLP data can therefore be used, at least in population Z3, for informed selection of individuals harbouring superior allele combinations of QTL-linked fAFLP markers based on the origin of the markers and direction of effects on the traits studied.

Studies on function mapping, SNP detection in the functional gene *Stp*23, QTL analysis using candidate genes offer new possibilities for future QTL analysis. On this basis, an improved strategy can be proposed. Since a large number of functional genes have been localized on the potato linkage map, functional genes can be readily selected based on the map positions and used as markers for QTL analysis for any traits of interest. As the genes are predominantly mapped by STS approach, the primer sets can be directly adopted from the present study to generate CAPS markers for the same genes in different populations provided that the same marker alleles are present. When sufficient SNPs are discovered for all these functional genes as done for *Stp*23, the genotyping process could be remarkably accelerated by automated detection and scoring of SNPs.

This strategy could offer many advantages as compared to fAFLP-tagging. First, since STS and SNP markers are potentially codominant in nature and could detect multiple alleles for a given locus, a more powerful program such as interval mapping could be applied to the data for QTL analysis. Second, since the map position of the genes are known, more detailed parameters of QTL, such as the locations, number and degree of effects and the interaction between QTL, could be addressed without constructing a linkage map. Third, when the biochemical basis of a trait under study is highly relevant to carbohydrate metabolism, the candidate gene approach can be used for QTL analysis as performed in the present study.

And finally, once significant QTL are identified, the functional gene markers associated with the trait could be incorporated directly into breeding programs for marker-assisted selection.

4.4.2 Magnitude of fAFLP-tagged QTL effects

Based on threshold set for declaring marker-trait associations, many significant QTL were identified in both locations. Some QTL were expressed in one location but not in the other. Some of these potential QTL may be false positives which resulted simply by chance. The frequency of false positives could be tested by permutation tests (Churchill and Doerge 1994) which were, however, not applied in this study. Nevertheless, when a fragment consistently showed association with a trait in independent trials with the same direction of effect or when a fragment showed associations with different but related traits, it was more likely that the fragment is linked to a genuine QTL. Similarly, a QTL detected in single environment may be caused simply by chance. However, considering the stringent threshold *P* value used, most QTL detected in only one location are likely to be the result of genotype x environment interaction.

The amount of variance explained by single fAFLP markers was small. The most significant marker accounted for 6 %, 12.6 % and 6.9 % of the variance of TSC, RSC and FE, respectively. This may be explained by several reasons. First, the genetic complexity of the traits which may be controlled by numerous genes. As described in Sect. 1.6, at least 70 genes are known to be involved in carbohydrate metabolism and transport and may potentially contribute to the biosynthesis of starch and sugars. Many genes may be responsible as QTL for the variation of TSC and RSC depending on the segregation population used (see Sect. 4.2.3). For FE, the knowledge of biochemical mechanisms underlying the trait is not well understood, however the polygenic nature is clearly demonstrated by its phenotypic distribution. There are reports indicating that 17, 6 and 7 QTL for TSC (Schäfer-Pregl *et al*. 1998), chip colour (reflecting the level of RSC) (Douches and Freyre 1994) and foliage maturity (Collins *et al*. 1999), respectively, have been identified in diploid mapping populations. Assuming that a similar number of QTL for TSC with additive effect were active in Z3, given the trait heritability of 59 %, it is conceivable that only 2 % - 6 % of the variation was accounted for by individual markers. Second, the marker density in the tetraploid population may not have been very high, due to the limited number of primer combinations used for marker generation. Consequently, recombination occurred between markers and QTL resulted in the reduction of the effect that can be detected by the marker.

Moreover, the dominant nature of the AFLP markers could not allow more precise classification of genotypes based on their allele configuration except the two classes with marker present or absent. Finally, the variation in trait measurement decreased the heritability of the trait.

4.4.3 Assignment of map positions of fAFLPs and segregation analysis

AFLP polymorphisms are defined by nine nucleotides (six nucleotides for the enzyme recognition sites plus three selective nucleotides) at the *Eco*RI end and seven nucleotides (four nucleotides for the enzyme recognition sites plus three selective nucleotides) at the *Mse*I end and the fragment size. It is likely that an AFLP fragment with the same size derived from different genetic backgrounds corresponds to the same locus. In contrary to RAPD markers which have an acute problem of transferability (Lynch *et al*. 1994; Black 1993), AFLP fragments mapped in one population can serve as chromosome-specific markers if they also segregate in another population. The transferability of AFLP markers has been demonstrated in barley (Waugh *et al*. 1997; Yin *et al*. 1999) and in potato (Li *et al*. 1998; Rouppe van der Voort *et al*. 1997). This observation provides the basis for inferring map positions of fAFLP markers derived from the tetraploid population Z3 from markers of identical size which are mapped in the reference mapping population K31.

fAFLP was performed on the reference mapping population K31 under the same conditions with the same primer combinations as performed in Z3. 43 fAFLP markers segregating in K31 were detected to be in common between the two populations K31 and Z3. Segregation data created by GeneScan® and Genotyper™ were integrated into the RFLP framework data set of K31 and analysed for linkages. 18 fAFLP markers were localised with a recombination frequency of less than 5 % to the nearest reference markers and multipoint analysis of recombination frequency allowed confident positioning of these markers (Figure 3-10). For the remaining markers, the recombination frequency with the closest reference markers was between 5 % - 18 % which, in most cases, may be overestimated. This may be due to the fact that the adapted threshold of peak quality 50 for fragment calling may not be fully fit for all the cases under investigation. Consequently, this may result in erroneous scores leading to the overestimation of the recombination frequency. Thus these markers were positioned only approximately.

The inferred map position of the fAFLP markers, although limited in number, could allow comparison of some fAFLP-tagged QTL with the ones previously detected in other studies. 17 QTL for TSC have been reported in K31 (Schäfer-Pregl *et al*. 1998). Several fAFLP-tagged QTL for TSC in Z3 were positioned in the similar regions. They are G3-393 (LG I), B3-206, G2-71 (LG II), B1-316, G3-342, *Sss*I (LG III), G1-90 (LG IV), B2-168 (LG V), B3-121 (LG IX), B3-130 (LG X), *Dbe*, B1-77 (LG XI), B1-103, B3-100, and G2-444 (LG XII). This finding suggests that these markers may have tagged the same gene or gene family controlling TSC as detected in the population K31. The QTL alleles are unlikely, however, the same since Z3 and K31 are genetically unrelated. The genetic variation of the QTL alleles in the two populations may be responsible for the potentially different degree of QTL effects.

Six QTL for chip colour (reflecting the level of RSC) have been revealed (Douches and Freyre 1994) with two on chromosome II, one on IV, two on V and one on X. Some fAFLP markers, were assigned to the same linkage groups such as G1-90 (LG IV), B3-215 (LG V), B3-141 (LG V), G1-342 (LG V), and B3-137 (LG V). Further comparison is not feasible due to the lack of common reference markers between the two populations investigated. Some fAFLP-tagged QTL for RSC were localized in the QTL regions for TSC in K31, such as B1-166 (LG I), G3-83 (LG I), *Stp*23 (LG III), G1-90 (LG IV), B2-398 (LG VI), G3-54 (LG IX), *Dbe* (LG XI), G2-89 (LG XI), B1-77 (LG XI), G1-169 (LG XII). The localization of QTL for different traits in a similar regions on molecular linkage maps may be due to pleiotropic effects of single genes on multiple traits. It cannot be excluded, however, that such results may be due to the linkage of two or more functionally independent genes. The lack of a high-resolution map prevents to distinguish these two possibilities.

7 QTL for FE have been identified with the one on LG V linked to marker GP179 being the most significant, accounting for up to 70 % of the phenotypic variation (Collins *et al*. 1999). The functional gene *Sut*2, which was closely linked to GP179, was selected for tagging the QTL in Z3 (see Sect.3.3.6). No effect was detected at this locus for FE in Z3. It should be noted that 5 fAFLP markers showing associations with FE in Z3 were clustered in an 18 cM interval flanked by RFLP markers GP179 and GP234. These multiple markers proximal to the region reinforce that the markers tagged a genuine QTL for FE. However, no fAFLP markers in this region showed such a notably significant effects as detected in the population analysed by Collins *et al*. (1999). One explanation is that QTL alleles in this population are different from that in Z3. It is also possible that the fAFLPs tagged a closely linked but

functionally different gene(s), or breeders' selection against very late genotype diminished the effects.

The inferred map position of the fAFLP markers represent only a small part of the markers showing associations with the analysed traits. More detailed QTL parameters in terms of location, number, interaction (epistasis), reproducibility and degree of effect, would rely on the availability of map positions of all other markers. This could be compensated using various reference mapping populations. An initial step has been made by Rouppe van der Voort *et al*. (1998) for AFLP mapping in potato by which a number of AFLP markers have been positioned on different linkage groups. When more and more AFLP markers with their map positions become available, it may become more feasible to infer the position of a AFLP marker from a reference AFLP map without cloning and sequencing.

The possible segregation type of fAFLP markers was determined based on a chi-square test for deviation from the segregation ratios of 1:1 and 1:5 expected for simplex- nullipex and duplex-nulliplex fragment configurations, respectively. Segregation ratios of 1:3, 1:11 and 1:35 are expected for parental genotype of simplex-simplex, simplex-duplex and duplex-duplex, respectively. 49 markers showed the expected segregation ratio as indicated in Tables 3-8, 3-9, 3-10. However, caution must be taken when reviewing the results since the method of inferring the mode of segregation type may result in a false hypothesis when segregation ratios are highly distorted. In most cases, distorted segregation ratios that were observed mainly favoured the undetected alleles (absence). Some may be due to the occurrence of double reduction which increases the production of homozygous gametes as compared to random chromosome segregation and leads to alteration of segregation ratios (Ronfort *et al*. 1998), others may be explained by the selection performed on the population by breeders. Distortion favouring the presence of a marker allele may also exist but cannot be tested reliably in the study because of the multiple alternative genetic models and the limited number of individuals available for testing, for example, goodness of fit for the ratio 1:35.

4.4.4 Genetic basis of correlations between phenotypic traits

A negative correlation (r=-0.62) was found for TSC and RSC in the population Z3. This relationship was also uncovered from QTL analysis. 15 marker loci were detected as having associations with both traits (Table 3-10). At each of these fAFLP loci, except G1-339, the alleles associated with increased TSC were associated with decreased RSC and *vice versa*.

This close association between QTL for TSC and RSC at these marker loci suggests that both traits may be controlled at least in part by the same gene(s) with pleiotropic effects.

Similarly, TSC and FE were also correlated with a correlation coefficient of 0.49 (see Sect. 3.3.2). Generally, high TSC is associated with delayed foliage maturity and *vice versa*. This was further confirmed in the QTL analysis by which 12 markers showed associations with TSC and FE. All markers except Y-393 and B1-244 had effects on both traits with direction congruent to their phenotypic correlation relationship, indicating that the same gene(s) with pleiotropic effects may be responsible for the QTL identified.

Fewer markers (7) have been detected to be associated with both RSC and FE, and the direction of effect on the two traits, unlike the previous two cases, was variable (Table 3-10). 3 markers were accordant in the direction of effect with the phenotypic correlation while 4 markers were contradictory. The variability of the direction may indicate the genetic complexity of the two traits. Some fAFLP may have tagged genetic factors with pleiotropic effects while others may detect functionally different but closely linked genes.

Overall, the marker-trait association analysis has revealed a number of fAFLP markers associated with single and multiple traits. The relationship between traits revealed by markers with multiple effects on the traits is consistent with their phenotypic correlations indicating that the fAFLP markers successfully tagged, at least in part, genetic factors contributing to the traits examined. The QTL analysis with fAFLP markers provide a genetic basis in terms of linkage or pleiotropy for the correlations observed between these traits. Briefly, the traits relationship between TSC and RSC, TSC and FE may be in part controlled by the same genetic factors with pleiotropic effects on different traits. It cannot be excluded, however, that functionally different but genetically closely linked genes are the cause of the correlations. Further work is required to get the definitive conclusions on this point.

The nature of pleiotropic effects of QTL on TSC and RSC was in agreement with the expectation of potato breeding for high TSC and low RSC. The partially common or closely linked genetic factors for TSC and FE imply that it may be difficult to increase TSC without delaying the foliage maturity through conventional breeding procedures. In this case, the use of molecular markers for the simultaneous improvement of TSC and FE may be more efficient than direct selection based on the phenotype. This could be achieved by pyramidizing favourable alleles for both traits at independent QTL.

4.4.5 Cloning and conversion of fAFLP markers and comparison of the two genotyping systems fAFLP and rAFLP

The marker-trait association analyses yield QTL being tagged by a number of fAFLP markers. The most significantly associated fAFLP markers need to be cloned and converted into easy-to-use markers for further investigation. To this end, a case study of cloning fAFLP fragments via radioactive AFLP was carried out using primer combination of B3. The experiment has demonstrated that it was possible to clone fAFLP markers via rAFLP approach. However, the efficiency of cloning was relatively low. There are reports indicated that successful cloning and conversion of AFLP markers was mostly limited to a range of smaller AFLP fragments (Reamon-Buettner and Jung 2000). In present study, cloning of fAFLP was bypassed using rAFLP. This technique change resulted in further decreasing of cloning efficiency since not all fragments could be re-identified unambiguously on autoradiograph. The conversion of the marker proved to be much more difficult than expected which could be attributed to the tetrasomic genome structure and the nature of the AFLP fragments. First, sequencing of ten clones revealed 7 nearly identical sequences which differed from one another by single nucleotides. The sequences were highly variable and the actual number of such sequences is likely more. Second, the polymorphism of the fAFLP fragment is based on the *Eco*RI and *Mse*I sites and the three selective nucleotides of the primers. Primers that are designed in any other region may result in lost of specificity defined by AFLP (Shan *et al*. 1999). Third, allele specificity revealed by sequence comparison between two parents could be useful for designing allele-specific primers for diploid inbred species but may not be so in highly heterozygous tetraploids like potato. This is further complicated by the fact that the cloned fragment detected multiple copies. Taking all these factors into consideration, conversion of fAFLP markers is a technically difficult and time-consuming exercise.

Cloning of fAFLP fragments via rAFLP also allowed the comparison of the two genotyping systems. Compared to rAFLP, fAFLP offers many advantages. (1) fAFLP is performed with fluorescence-labelled primers in combination with DNA sequencer, thus precluding the necessity of working with radioactivity. (2) Since primers are labelled with different fluorescent dyes, fAFLP products could be mixed and loaded in one lane on the gel, thus significantly reducing the number of gels to be run per study. (3) Electrophoresis of fAFLP is viewable directly on the gel image at real time, and the process can be completed within 4 hours using appropriate running parameters. The results that can be subjected to down stream

computer-aided analysis eliminating all steps like fixing, drying and exposure as required by rAFLP analysis. (4) Highly precise sizing with single base pair resolution and data output both qualitative (scored as presence and absence) and quantitative (scored as peak height) in *Excel* format can be achieved semi-automatically by GeneScan® and Genotyper™ software eliminating the possibility of errors occurring during manual data handling. (5) fAFLP generates fragments with a larger scorable size range from 50bp to 500bp or more if needed, while fragments out side the range of 100-500 bp were hardly scorable on autoradiographs. (6) fAFLP is extremely sensitive and powerful in detecting trace amount of product while for rAFLP, the trace products appear as faint bands which vary with the exposure time and the decay period of radioactivity of the label used. Therefore, fAFLP generated much more scorable fragments than rAFLP. For example, a total of 117 scorable fragments were generated with primer pair E-act/M-cta using the automated fluorescent AFLP system, whereas only 72 fragments could be clearly identified on autoradiograph using radioactively labelled AFLP technique. This discrepancy is largely due to the faint bands that could hardly be unambiguously scored on autoradiographs. Similar observations were also reported by Zhang *et al*. (1999).

While having these advantages, fAFLP technology has also inherent problems. Since it is extremely sensitive and detects even trace amounts of product, it can also easily generate environmental noise resulting from either the overlapping of the wavelengths between different colour dyes or the contamination of gel plates. To minimize the influence on the results, a quality threshold of 50 was selected for fragment scoring. In other words, only fragments that were higher than 50 in peak height were scored. In my experience, such threshold seemed optimal although it was chosen arbitrarily. Moreover, the overlapping of the wavelength between different dyes can cause spectral interference between the dye labels during analyses. Consequently, the size standard definition derived from one of the samples may not fully fit to those of others, which, when not verified, may result in an erroneous assignment of size to fAFLP fragments. Therefore, the size standards of all the samples have to be visually checked to eliminate this possibility before proceeding to data analysis with Genotyper™. In genotyping using Genotyper™ software, fragment calling is performed by converting fragment size into prior-defined size categories. In the present study, the range of size categories was limited to a single base pair. As a result, the same-size fragments may be split into two different ones in some cases due to slight lane-to-lane variation. A post viewing of the output data is also required to minimize such errors. Finally, recovering of fAFLP

fragments of interest cannot be performed on the system itself to date but has to rely to rAFLP. Due to the difference of sensitivity of the two genotyping systems, rAFLP could not detect all the fragments which are identified on the fAFLP system.

4.4.6 Function map-based QTL analysis

The candidate gene strategy has been shown to be very useful and effective in the analysis of genetic factors controlling phenotypic traits with a known biochemical and physiological basis (Winkelman 1992, Causse *et al*. 1995, Faris *et al*. 1999). The basis of the candidate gene strategy is that relationships between functional genes and traits are known and that genome regions showing a QTL effect in one population also show marker-QTL associations in the same region in other populations (Oberhagemann *et al*.1999, Kearsey and Farquhar 1998). In potato, the function map for genes involved in carbohydrate metabolism (see Sect. 3.1) provides the framework for the application of candidate gene strategy in the QTL analysis for starch and RSC. Five functional genes selected on the basis of known overlapping positions with QTL for TSC identified in population K31 (Schäfer-Pregl *et al*.1998) were tested for association with phenotypic traits in population Z3. Association analysis indicated that genes *Dbe* and *Sss*I were significantly associated with QTL affecting TSC in population Z3 at the 1 % level. No significant effect was detected at the other gene loci. More prominent results were obtained for RSC. Two loci *Stp*23 and *Sut*2, on chromosomes III and V, respectively, showed significant effects consistently detected at two locations at 1 % level. It is worth noting that *Stp*23 is closely linked to *Pain*-1 (coding for soluble acid invertase, see Figure 3-3 on page 57) which, based on its functional role, appears more likely to be the candidate for the QTL controlling RSC. The effects observed at *Stp*23 may result from the linked *Pain*-1 gene. Gene *Sut*2, a member of sucrose transporter family, was repeatedly detected as a QTL affecting RSC. The observation is in accordance with the putative physiological role of *Sut*2, which is believed to act as sucrose sensor to control sucrose fluxes across the plasma membrane of sieve element (Barker *et al*. 2000). It should be noted that a major QTL for FE was identified at RFLP locus GP179 (Oberhagemann *et al*. 1999, Collins *et al*. 1999), however, no such effects were detected at the *Sut*2 locus, which is closely linked to GP179, in the population Z3. The possible explanations may be that the effects were not large enough to be detected in the experiment with the statistical method used or alleles were homozygous at putative QTL loci or a PCR-based assay at locus *Sut*2 may have detected a allele ineffective on the trait.

5. SUMMARY

5.1 Function mapping as an approach to the identification of genes controlling tuber starch content in potato

Carbohydrates are the major storage compounds of cereal and tuber crops. In potato, tuber starch content, which is a quantitative character, is of agronomic importance and is controlled by genetic factors and environmental conditions. Genetic mapping using anonymous RFLP markers allowed the dissection of the genetic component of the quantitative trait into discrete quantitative trait loci (QTL) and their localization on the potato molecular map. QTL analysis alone does not reveal the identity of the gene(s) controlling the trait at the molecular level. Molecular linkage maps based on functional gene markers may shed light on this issue by correlating the relevant functional genes to QTL for the trait, thus leading to the identification of candidate genes underlying a quantitative trait. For this purpose, the nucleotide sequence databases EMBL and GenBank were explored for sequence information of genes from potato, tomato and other plant species. Priority was given to genes operating in carbohydrate metabolism and transport. Sequence information was used to amplify the homologous or analogous genes in potato by PCR. Polymorphisms of the amplification products were revealed either directly or after cleavage with restriction enzymes in two diploid mapping populations K31 and LH. Reliability of the mapping approach was confirmed by sequence comparison of the PCR products with the source genes and by comparing map positions of PCR products with corresponding loci previously determined by RFLP. A potato function map consisting of 82 loci was constructed based on 66 cloned genes of known biochemical function. The comparison of the molecular function map with the QTL map for tuber starch content, which was previously constructed using the same population, revealed a number of correlations between the map positions of QTL for tuber starch content and loci of function-related genes. 14 out of 17 QTL for tuber starch content were linked to at least one known candidate gene locus. The clearest positional correlation was found between the *Stp*23 gene encoding starch phosphorylase and QTL *ts(g)* on linkage group III.

The gene markers, as defined in this study, are a valuable tool for genetic analysis and marker-assisted selection since they are predominantly PCR-based and cover a considerable

proportion of the potato genome. To enhance further the efficiency of genotyping a population for a given functional gene, it is highly desirable that the gene markers are amenable to automatization. To this end, candidate gene *Stp*23 was selected as a case study for exploring single nucleotide polymorphisms (SNPs), which have much potential for automated detection and scoring. PCR products of 14 potato lines were sequenced. Bi-allelic and tri-allelic SNPs were revealed within single lines and sequence assembly of different lines revealed potential SNPs existing in potato genotypes. Together, 31 candidate SNPs were identified in a 1801 bp segment of *Stp23*, 8 of which were in coding regions and 23 were in introns.

5.2 fAFLP tagging of genetic factors controlling tuber starch content, reducing sugar content and foliage earliness in tetraploid potato

Tuber starch content, reducing sugar content and foliage earliness are three important agronomic traits in potato breeding program. QTL analysis for the traits has been addressed in a few studies using diploid potato. While providing insight into the genetic nature of the trait studied, studies performed on diploids may suffer from the disadvantage of ploidy manipulation and difference in QTL effects may exist at two ploidy levels (Groose *et al*. 1988). Therefore in the second section of the study, a QTL analysis was carried out in collaboration with a breeding company in a tetraploid hybrid family Z3. An efficient fAFLP genotyping system performed on a 377 DNA sequencer was adopted for DNA marker generation. A total of 806 fAFLP markers were tested for association with QTL for tuber starch content, reducing sugar content and foliage earliness. 40, 25, 32 fAFLP fragments were identified to be associated with tuber starch content, reducing sugar content and foliage earliness, respectively. 33 fAFLPs showed associations with two or more traits.

To obtain positional information of these fAFLP markers on the potato map, the reference mapping population K31 was also genotyped in parallel with the same system and using the same primer combinations. Map positions of 50 fAFLP fragments were inferred from common markers mapped in the reference mapping population K31. Nine were located in map regions of previously identified QTL for tuber starch content.

Summary

For breeding-oriented QTL analysis, the most significantly associated markers are required to be cloned and converted into a marker type that is amenable to marker-assisted selection. To achieve this goal, radiolabelled AFLP was used for cloning fAFLP fragments of interest. However, cloning and conversion of fAFLP fragments via rAFLP was not efficient due to the difference in sensitivity between the two genotyping systems and the complexity of tetrasomic inheritance of tetraploid potato. Only one fragment, which was highly associated with reducing sugar content, was cloned and sequenced but failed to be converted into a 'easy-to-use' marker.

With the availability of the potato function map described in the first section, it become feasible to perform QTL analysis via a candidate gene approach for any given population. To test this, five candidate genes, which overlapped with QTL for tuber starch content on the map of K31, were selected to test for association with the traits investigated in population Z3. Genes *Stp*23 (starch phosphorylase) and *Sut*2 (sucrose transporter like) detected significant associations with reducing sugar content at two different locations. Three alleles of the gene *Dbe*, coding for a debranching enzyme, had an effect on tuber starch content at one location.

5. ZUSAMMENFASSUNG

5.1 Funktionelle Kartierung als ein Ansatz zur Identifikation von Genen, die den Stärkegehalt der Kartoffelknolle kontrollieren

Kohlenhydrate sind in Getreidepflanzen und knollenbildenden Feldfrüchten die dominierenden Speicherverbindungen. Der Stärkegehalt der Kartoffelknolle ist ein quantitatives Merkmal von agronomischer Bedeutung und wird durch genetische Faktoren und Umwelteinflüsse reguliert. Dieses quantitative Merkmal konnte durch genetische Kartierung mit Hilfe von anonymen RFLP Markern in diskrete QTLs zerlegt werden, die auf der genetischen Karte der Kartoffel lokalisiert wurden. Die QTL-Analyse allein führt hingegen nicht zur Identifizierung der merkmalskontrollierenden Gene auf der molekularen Ebene. Hier können molekulare Kopplungskarten hilfreich sein, die auf funktionellen Gen-Markern basieren. Die Korrelation von Merkmals-relevanten Genen mit QTLs für dieses Merkmal kann zur Identifikation von Kandidatengenen führen, die das quantitative Merkmal prägen. Zu diesem Zweck wurden die Sequenzdatenbanken EMBL und GenBank vorwiegend nach Kartoffel- und Tomatengenen, aber auch Genen anderer Pflanzen durchsucht, die dem Kohlenhydratstoffwechsel und -transport zuzuordnen sind. Von der Sequenzinformation dieser Gene wurden Primer abgeleitet, mit deren Hilfe homologe bzw. analoge Sequenzen aus der Kartoffel mittels PCR amplifiziert wurden. Segregierende Polymorphismen zeigten sich nach der Amplifikation von DNA der verschiedenen Pflanzen der diploiden Kartierungspopulationen K31 und LH, entweder direkt oder nach dem Restriktionsverdau der PCR-Produkte. Die Verläßlichkeit dieses Kartierungsansatzes wurde durch Vergleich der Nukleotidsequenz der PCR-Produkte mit der Datenbanksequenz, von der die Primer abgeleitet worden waren, und durch Vergleich der Positionen der gewonnenen PCR-Produkte auf der Genkarte mit den entsprechenden bereits vorher mittels RFLP bestimmten Loci überprüft. Hierdurch wurde eine funktionelle Genkarte der Kartoffel mit 82 Loci basierend auf 66 klonierten Genen von bekannter biochemischer Funktion erstellt. Die QTL-Karte des Kartoffelgenoms bezüglich des Stärkegehalts der Kartoffelknolle, welche von derselben Population konstruiert worden war, konnte nun mit der funktionelle Genkarte verglichen werden. Es wurde deutlich, daß sich eine bedeutende Anzahl an Positionen aus den beiden verschiedenen Kartierungsansätzen überlagerten. 14 der 17 QTLs konnten mindestens einem Locus für ein bekanntes Kandidaten-Gen zugeordnet werden. Die beste positionelle

Korrelation fand sich zwischen dem *Stp*23-Gen, das für eine Stärkephosphorylase codiert, und dem QTL *ts(g)* auf Chromosom III.

Die von bekannten Genen abgeleiteten Marker, wie sie in dieser Arbeit vorgestellt werden, sind ein nützliches Hilfsmittel für die genetische Analyse und für die Marker gestützte Selektion, da sie hauptsächlich auf PCR-Techniken basieren und einen beträchtlichen Teil des Kartoffelgenoms abdecken. Zur Steigerung der Effizienz der genotypischen Analyse einer Population bezüglich eines gegebenen funktionellen Gens ist es wünschenswert, den Gen-Marker einer automatischen Analyse zugänglich zu machen. SNPs (single nucleotide polymorphisms) eignen sich besonders gut für eine automatische Detektion und Auswertung. Beispielhaft wurden hierzu PCR-Produkte des Kandidaten-Gens *Stp*23 von 14 Kartoffellinien sequenziert. Innerhalb einzelner Kartoffellinien wurden bi- und triallelische SNPs gefunden. Der Vergleich der Sequenzen verschiedener Genotypen führte zur Identifizierung potentieller, für die Automatisierung geeigneter SNPs. So wurden insgesamt 31 SNPs in einem 1801 bp langen Segment des *Stp*23-Gens identifiziert von denen 8 in der codierenden Region und 23 in den Introns zu finden waren.

5.2 fAFLP-Tagging von genetischen Faktoren, die den Stärkegehalt, den Gehalt von reduzierenden Zuckern der Kartoffelknolle und die Reifezeit kontrollieren

In der Kartoffelzüchtung sind der Stärkegehalt der Knollen, der Gehalt an reduzierenden Zuckern bei der Lagerung und die Reifezeit Merkmale von herausragender Bedeutung. Einige Studien widmen sich der QTL-Analyse dieser agronomischen Merkmale unter Verwendung von diploiden Kartoffellinien. Diese liefern zwar einen aufschlußreichen Einblick in die genetische Natur der untersuchten Merkmale, reichen jedoch nicht zur Beschreibung von QTL-Effekten bei tetraploiden Kartoffellinien. Deshalb wurde im zweiten Teil dieser Arbeit in Zusammenarbeit mit einem Kartoffelzuchtunternehmen eine QTL-Analyse einer tetraploiden Hybridfamilie Z3 unternommen. Zur Gewinnung von DNA-Markern wurden auf effizient Weise fAFLP Produkte mit einem 377 DNA-Sequenzierer analysiert. Insgesamt wurden 806 fAFLP Marker auf ihre Kopplung mit QTLs für den Stärkegehalt der Kartoffelknolle, den Gehalt an reduzierenden Zuckern und die Reifezeit hin untersucht. Davon waren 40 fAFLP Marker mit QTL für den Stärkegehalt, 25 mit QTL für

den Gehalt an reduzierenden Zuckern und 32 mit QTL für die Reifezeit assoziiert. 33 fAFLP Marker waren mit zwei oder mehr Merkmalen gekoppelt.

Um positionelle Information über diese fAFLP Marker auf der genetische Karte der Kartoffel zu erhalten, wurde auch die diploide Kartierungspopulation K31 mit derselben Technik unter Verwendung der gleichen Primerkombinationen untersucht. 50 fAFLP Fragmente waren mit bereits bekannten RFLP-Markern in der Referenzpopulation K31 gekoppelt. 9 fAFLP-Fragmente wurden in Regionen lokalisiert, die zuvor als QTLs für den Stärkegehalt der Kartoffelknolle identifiziert worden waren.

Will man die signifikantesten Marker für eine QTL-Analyse in einem Züchtungsprogramm nutzen, so müssen diese kloniert und in einen Markertyp umgewandelt werden, der in der Marker-gestützten Selektion leicht zu handhaben ist. Hierzu wurden fAFLP-Fragmente in radioaktiv markierte rAFLP-Fragmente überführt. Es stellte sich allerdings heraus, daß die Klonierung und Umwandlung von fAFLP-Fragmenten über die Herstellung von rAFLP-Fragmenten nicht effizient genug ist. Einerseits ist die Sensitivität der beiden Markierungstechniken der AFLP-Fragmente zu unterschiedlich, andererseits begrenzt die Komplexität der Vererbung der genetischen Faktoren bei tetraploiden Kartoffeln eine problemlose Umwandlung aller fAFLP-Fragmente in radioaktiv markierte, klonierbare AFLP-Produkte. Allerdings gelang es, ein AFLP-Fragment, das hoch signifikant mit dem Gehalt an reduzierenden Zuckern gekoppelt war, zu klonieren und zu sequenzieren. Ein einfach zu handhabender Marker konnte von diesem Fragment jedoch noch nicht abgeleitet werden.

Mit der Verfügbarkeit einer funktionellen Karte des Kartoffelgenoms, so wie sie im ersten Abschnitt beschrieben wurde, ist es möglich, eine QTL Analyse mittels Nutzung der Sequenzinformation von Kandidaten-Genen für jede beliebige Population durchzuführen. Zum Test wurden fünf Kandidaten-gene, die mit QTLs für den Stärkegehalt der Kartoffelknolle in der Population K31 gekoppelt sind, auch in der Population Z3 auf Assoziation mit dem Stärkegehalt hin untersucht. Drei Allele des *Dbe*-Gens, das für das "debranching"-Enzym kodiert, zeigten einen Effekt auf den Stärkegehalt. Die Gene *Stp*23 (Stärkephosphorylase) und *Sut*2 (Saccharosetransporter-ähnlich) zeigten eine signifikante Kopplung mit dem Gehalt an reduzierenden Zuckern.

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7. APPENDIX

Six new nucleotide sequences of functional genes in potato*

7.1 Partial potato genomic sequence of gene *Rca*[a]

```
  1  ACACCGTCAA  CAACCAGATG  GTGAATGCCA  CCCTCATGAA  CATTGCTGAC
 51  AACCCAACAA  ATGTCCAGCT  CCCCGGTATG  TACAACAAGC  AAGAGAACTG
101  CAGGGTCCCC  ATTATTGTCA  CTGGTAACGA  CTTCTCCACA  TTGTATGCTC
151  CTCTTATCCG  TGATGGTCGT  ATGGAGAAGT  TCTACTGGGC  ACCAACTAGG
201  GAGGATAGAA  TTGGTGTTTG  CAAGGGTATT  TTCAGAACTG  ACAACGTGCC
251  TGAGGAAGCT  GTTGTAAAGA  TTGTCGATTC  CTTCCCTGGA  CAATCTATTG
301  GTACAAACAC  TAAAGAATTC  AAAATGAAAT  TTCTCTTTTA  A
```

7.2 Partial potato genomic sequence of gene Ant[a]

Sequence I

```
  1  TGGAGAGGAA  ACACTGCCAA  TGTTATCCGT  TATTTCCCCA  CTCAGGTTTA
 50  CCATTTGTCT  TCATTTTGAG  GGCTGTAATT  TATGCATCAA  AGATACATGC
101  ACATTTATTG  CTCTGTTGTT  TGCTTAAGTC  GAGCATTGAC  NGACTGATGC
                                                    (C/T)
151  TCTGTTCCAC  AGGCCCTGAA  CTTTGCATTC  AAGGACTACT  TCAAGAGACT
201  CTTCAACTTC  AAGAAGGACC  GTGATGGCTA  CTGGAAGTGG  TTTGCTGGCA
251  ACCTTGCCTC  AGGTGGTGCT  GCTGGTGCTT  CTTCTTTGTT  CTTTGTCTAC
301  TCCTTGGACT  ATGCTCGTAC  CCGTCTTGCT  AATGACGCCA  AGGCTTCAAA
351  GAAGGGAGGT  GAGAGGCAGT  TCAATGGTTT  GGTTGATGTC  TACAAGAAGA
401  CACTTAAATC  TGATGGAATT  GCTGGTCTAT  ACCGTGGATT  CAACATTTCA
451  TGTGTTGGTA  TCATTGTTTA  CCGTGGTTTN  TACTTTGGAA  TGTACGACTC
                                (A/G)
501  CTTGAAGCCT  GTCCTCTTGA  CAGGAAACCT  GCAGGTTAGT  GTTTTTATAA
551  TGTTGGCTTT  GCTATGCTTG  TTCA
```

Sequence II

```
   1  CCTGGGTTGT TCCTGTTCCA TTTTTTTAAA GTATGGAATG CTGGTTAATA
  51  TTGTGTTCCA CAGGCATTGA ACTTTGCATT TAAGGACTAC TTCAAGAGGC
 101  TCTTCAACTT CAAGAAGGAC CGCGATGGCT ACTGGAAGTG GTTTGCAGGT
 151  AACCTGGCAT CTGGTGGTGG TGCTGGTGCT TCCTCTTTGC TCTTTGTTTA
 201  CTCCCTTGAC TATGCTCGTA CTCGTCTTGC AAATGATGCC AAGGCTGCAA
 251  AGAAGGGAGG TGGTGGCAGA CAATTCGATG GGTTGGTTGA TGTCTACAGA
 301  AAGACACTTA AATCTGATGG AGTTGCTGGC TTGTACCGTG GGTTTAACAT
 351  TTCATGTGTT GGTATCATTG TGTACCGTGG TTTGTACTTT GGAATGTATG
 401  ATTCATTGAA GCCAGTGCTG TTGACTGGAA AGATGGAGGT TAGTTATCCA
 451  TCTATTTTTG GTATTTTCCC ATATTGCTAG TTTGCTATTT TGTGATGATA
 501  TCATATGTTG ATAGTTATGT TCAGATGCTT ACAATACTTG GAGATTGTTG
 551  CCTGTTACTT TGGAGTTGGG TTTTAGGATG TTATGGTTGG AATATGACTT
 601  CATTCTATTT TTTTATTNTC TTGATGGTTG CAGGATAGTT TCTTTGCTAG
                    (C/T)
 651  CTTTGCTCTT GGATGGCTCA TCACCAATGG TGCTGGTCTT GCATCGTACC
 701  CTATTGACAC AGTTAGGAGA AGAATGATGA TGACATCTGG TGAGGCAGTG
 751  AAGTACAAGA GCTCGTTCGA TGCATTCAAC CAAATCCTTA AGAATGAGGG
 801  TCCCAAATCA CTCTTCAAGG GAGCTGGTGC CAACAT
```

7.3 Partial potato genomic sequence of gene *Ndps*[a]

```
   1  ATGATCAAGC  CTGATGGTGT  CCAGTGGCCT  GGTTGGTGAG  ATTATCGGCA
  51  GATTTGAGAA  GAAAGGATTT  TCTTTGAAAG  GCTTGAAGCT  CATCACTGTG
 101  AACCATGCCT  TTGCTGAGAA  GCATTACGCT  GACTTGTCTG  CTAAGCCTTT
 151  CTTTAATGGG  CTTGTTGAGT  ATATTGTTTC  TGGACCTGTT  GTTGCTATGG
 201  TCTGGGAGGG  TAAGGGTGTA  GTTGCCACTG  GCAGGAAGAT  CATTGGAGCA
 251  ACCAACCCCT  TGGAGTCGGC  TGCTAGTACC  ATCCGTGGTG  ATTTTGCTAT
 301  TGATATTGGC  AGGAATGTTA  TTCATAGAAG  TGATGTTGTT  GAGAGTGCTA
 351  GGAAGGAGAT  TGCTTTTTGG  TTCCCCGAAG  GAATTGCAGA  GTGGCAGAGC
 401  AGCCTTCACT  CTT
```

7.4 Partial potato genomic sequence of gene *Eno*[b]

```
  1 AGCTGTTGTT  AGATAATCTG  GTGGCTGAGA  TGATTTTCTG  TTTAGGTGGT
 51 CATTGGAATG  GATGTTGCAG  CATCTGAATT  TTACGGAAAG  GACAAATCTT
101 ATGACCTGAA  CTTCAAAGAA  GAGGCAAGTA  CTGACTATTG  CTTCTTTCAC
151 ACATGCTTTT  CCGAGGTAGT  CTCGTAGTCA  AACTGTTCAT  ACGTTATTCA
201 GTTTTGCTCA  TATTTATTCT  TATTTGCAGA  GCAATGACGG  CTCACAAAAG
251 ATATCAGGTG  ACCAACTCAA  GGATTTGTAC  AAGTCATTTG  TGTCCGAGTA
301 CCCTATTGTT  TCAATTGAAG  ATCCATTTGA  CCAAGATGAC  TGGGAGACCT
351 ATGCTAAGCT  CACCACTGAG  ATTGGGGAGC  AAGTACAGAT  TGTCGGAGAT
401 GATCTCCTTG  TCACCAACCC  TAAGGTAAGA  TTGAAGCTTT  AACAATATTG
451 CTTGCATATC  AGAGTTGCTG  TTTCCATTGA  AAAGAAAAGC  AGACATCAAT
501 TTCTCTAGGT  GTTGATGTTT  ACCATGTATA  CTTATTTATA  ATGGTGTGCA
551 GAGGGTCGCC  AAGGCAATTG  CAGAGAAGAC  TTGCAATGCT  CTTCTTCTCA
601 AGGTATCATC  CTATCCAGCT  TATAGGTTGT  ATATTTCCGC  CATGATAATG
651 AGAGTTTCCT  CTTTGAGATG  TTATTAAATC  ATTTCTCTCT  CGATATGTGT
701 CAGGTTAACC  AAATTGGCAG  TGTGACCGAG  AGTATTGAAG  CTG
```

7.5 Partial potato genomic sequence of gene *Glo*[b]

```
  1 CTGATTTTCT  CCATTCATGC  TGTTGTTCCA  GGTTGTGAAA  GGGCACAAGG
 51 CAGGATCCCT  GTATTCTTGG  ATGAAGGTGT  CCGCCGTGGA  ACAGATGTCT
101 TTAAAGCTTT  GGCACTTGGA  GCTTCAGGCA  TTTTTGTAAG  TACCATATAA
151 TGATTGGACC  TAGATTGGTG  GCACATAGCT  CAATCTTTTA  TCTTTATCTA
201 CTCACCAGTA  GACCAAGTAG  TGCCTTTTTC  CAAAATCTGC  CTTGAGAATG
251 GTCAACAATC  TGAAAGTGAT  TTTAAATCTC  CCTAATAGTC  GTAGTAATAC
301 AATAATTACG  CCTCAGTCCA  AAGCAAGTTT  AGTATCTTCC  CTTTTAAGCC
351 GCTATATTGG  GAAATTCAAA  GTTAATAGAA  GTCCCTTGCA  CTTTCGAGAA
401 CCAAGAAAAG  TAGGCATTAT  AATTTATACC  TACATAATCA  GTGAATGATA
451 AATAACAGCT  GGTAAAATAT  TGGAGAACAA  GTTCCATGGG  TAAATGGGAA
501 ATGGGACAGT  CTTAACACAT  TCCAAAACCA  GTTCATTGTT  GGCTAAGCAC
551 ACAGTTACAT  ATTATGCCAT  GTTTCCTTTT  CGTCTTCAAA  CATCTTAGCG
601 AGTGACATTC  TGTATTGTCC  TTAACAGATT  GGAAGGCCAG  TAGTTTTCTC
651 ATTGGCTGCT  GAAGGAGAAG  CTGGAGTCAA  AAAAGTGCTG  CAAATGTTGC
701 GCGATGAGTT  TGAGCTAACT  ATGGCATTGA  GTGGCTGTC
```

7.6 Partial potato genomic sequence of gene *Pgk*[a] (see Figure 3-4 on page 59)

* Italics residues indicate intron; primer sequences were underlined; 'N' represents potential bi-allelic SNP with alternative nucleotides given in parentheses.

[a] Sequence data based on PCR product as template

[b] Sequence data based on cloned PCR product as template

Rca= ribulose bisphosphate carboxylase activase, *Ant*= adenylate transporter, *Eno*= enolase, *Ndpk*= nucleoside diphosphate kinase, *Glo*= glycolate oxidase, *Pgk*= phosphoglycerate kinase

ERKLÄRUNG

Ich versichere, daß ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit – einschließlich Tabellen, Karten und Abbildungen -, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; daß diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; daß sie – abgesehen von unten angegebenen Teilpublikationen – noch nicht veröffentlicht worden ist sowie daß ich eine solche Veröffentlichung vor Abschluß des Promotionsverfahrens nicht vornehmen werde.

Die Bestimmungen dieser Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Priv.-Doz. Dr. Christiane Gebhardt (MPIZ, Köln) betreut worden.

Köln, den 24. 08. 2000

CHEN Xinwei

Teile dieser Arbeit sind in folgenden Veröffentlichungen enthalten

Xinwei Chen, Ralf Schaefer-Pregl, Josef Strahwald, Francesco Salamini and Christiane Gebhardt (2000) Function map as an approach to identification of genes controlling tuber starch content in potato cultivars. Plant and Animal Genome VIII (PAGVIII), January 9-12, 2000, San Diego, USA

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