Genetic analysis of potato tuber quality traits

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Chapter 1

General introduction: Breeding for quality in potato

Jeroen S. Werij

Abstract

Potato is number three in the top five crops in terms of global food production. Increasing yields are important in providing food security for a growing world population. However, improving potato tuber quality traits and addressing specific demands made by consumers and processing industry can achieve equal benefits for human health and nutrition. So far, over 60 quality traits (such as tuber morphology, cold sweetening, starch and protein characters) from potato were mapped on all twelve potato chromosomes. With the availability of the genome sequence it will be possible to actively search for the underlying genes and develop suitable markers for these traits In order to achieve these goals it is of utmost importance to use all the knowledge available of natural variation, the genetic processes regulating quality and the development of tools to identify and select for the genes of interest. A future challenge will be to integrate all this knowledge in a genomics-assisted breeding strategy aiming to create new and improved varieties with outstanding quality.

An introduction to quality

Until now, high yield has been the main goal for the plant breeding industry worldwide. Every newly introduced variety should have an improved level of production over the existing varieties and, next to the yield, an improved agronomic performance. The latter improvements are made in different areas, ranging from the introduction of resistance to pests and diseases, to tolerance to abiotic stress factors like salinity, drought or low mineral content of soils. Further improvements can be made in physiological and plant architectural traits leading to improved agricultural performance. An additional area of improvement comprises quality aspects and it is an area that is likely to become ever more important in the near future.

Yield and disease resistance will always stay important traits to improve, especially in assuring a stable food supply for the growing world population. However, in those parts of the world where food availability is not an issue, simply producing a higher yield is no longer enough. There are several reasons why the focus is shifting more towards breeding for quality depending on the perspective of the target market. For instance, from a practical growers perspective, being able to produce outstanding quality can offer a distinctiveness that can create extra revenue (Caswell and Mojduszka, 1996). The physical properties connected to general quality traits, like shape, colour and size, are easily observable by consumers and are therefore major factors determining market value of a product. For their commercial success, breeders have to take all the demands into account from crop producers, processors, distributors and consumers while at the same time realise that "quality" is a continuously changing concept for each of the parties involved in the process.

In this review the different aspects of quality will be discussed, from traits to their genetics to breeding techniques, with a focus on potato (*Solanum tuberosum*). Potato ranks third in terms of global food production according to recent FAO statistics and is thereby the most important non-cereal food crop, grown throughout the world (Table 1). There are nearly 125 countries were potato is grown and it is consumed by a billion people on a daily basis (Mullins et al., 2006). The year 2008 was declared by the United Nations as the "international year of the potato", affirming the need for focus on the role that the potato can play in providing food security (United Nations General Assembly Resolution, 2006). Nutritional content and limiting losses in the processing industry are clear examples indicating that food security is a key factor for the increasing importance of quality from a breeding point of view. In this review an overview of the research is presented on a broad spectrum of potato tuber quality traits with a focus on the genetics behind these traits. Furthermore an insight is given in the genetic principles of quality and the use of modern breeding tools to get the observed variation in new varieties.

	harvested area	quantity	yield
Africa	1541498	16706573	10.8
Asia/Oceania	8732961	137343664	15.7
Europe	7473628	130223960	17.4
Latin America	963766	15682943	16.3
North America	615878	25345305	41.2
WORLD	19327731	325302445	16.8

Table 1: World potato production by region in 2007. Harvested area is in hectares, quantity in tonnes and yield in tonnes per hectare (FAOSTAT)

Quality traits of fresh potatoes

Colour, size and shape are crucial quality aspects for consumers in buying vegetables, as they are immediately obvious while making the purchase. This aspect resulted in an extensive research over the last century to understand how these traits develop. One of the most easily noticeable traits of potato tubers is the skin colour. This can range from whitish yellow, orange, brown and red to deep purple (Figure 1). The accumulation of anthocyanin pigments in the phelum and epidermis layers of the tuber is primarily responsible for the skin colouration. The earliest report mentions three loci affecting skin colour in tetraploid potato (Salaman, 1910). The three loci are named D (developer, known as I locus in diploid potato), required for tissue-specific accumulation of anthocyanin in tuber skin, R (red) required for the production of red anthocyanins and P (purple) for the production of purple pigments. Red pigments have been shown to be derivatives of pelargonidin and the purple anthocynanins are derived from delphinidin (Lewis et al., 1998; Naito et al., 1998; Rodriguez-Saona et al., 1998). D, R and P have been localized on the potato chromosomes 10, 2 and 11 (Gebhardt et al., 1989; Van Eck et al., 1994b; Van Eck et al., 1993). By genetic and transgenic studies it was confirmed that dihydroflavonol-4 reductase (dfr) is the active gene at the R locus (Zhang et al., 2009). The P locus encodes for flavonoid 3',5'-hydroxylase (*f3'5'h*) (Jung et al., 2005) and the D locus harbours a R2R3 MYB transcription factor that co-ordinately regulates the expression of anthocyanin biosynthetic genes (Jung et al., 2009). Some of the genetics underlying skin colour revealed a broad gene function that also affects other related traits. In this case, the MYB gene from the D locus also has a function in conferring purple tuber flesh colour by regulating anthocyanin accumulation in tuber flesh tissue (Rommens et al., 2008a). However, a recent publication indicates that anthocyanin production may be more complex than the previously described system with the D, R and P locus. In a microarray experiment conducted on pigmented and non-pigmented tuber samples no less than 27 genes were differentially expressed, including a novel single domain MYB transcription factor (Stushnoff et al., 2010). Although anthocyanins

do confer red or purple flesh colour, outside the origin of cultivated potato in the South American Andes it is rare to find cultivars with anthocyanin pigments in the tuber flesh.

Figure 1: An overview of different skin colours in potato

In cultivated potato, the flesh colour is predominantly white or yellow. Strong cultural preference exists for either white, preferred in the US and UK, or yellow, for instance in the Netherlands and Germany, making breeding for potato flesh colour important. Figure 2 shows an example of white and yellow flesh colour. The yellow to orange colouring of the potato tuber flesh is caused by the presence of certain carotenoids. Lutein, violaxanthin, zeaxanthin and antheraxanthin are the forms predominantly present in cultivated potato (Breithaupt and Bamedi, 2002; Brown et al., 1993; Iwanzik et al., 1983; Nesterenko and Sink, 2003). Next to the colouration, carotenoids are also reported to have a wide variety of human health benefits, but these attributes will be discussed later on in this review. Yellow flesh colour is caused by a dominant allele at the Y (yellow) locus, which was mapped on chromosome 3 of potato (Bonierbale et al., 1988). On the same location as the Y locus a beta-carotene hydroxylase gene (*bch* or *Chy2*) was mapped, thereby indicating that this is the most important candidate gene for yellow flesh (Thorup et al., 2000). The gene *Chy2* was confirmed to be the controlling factor for flesh colour by expression and SNP analysis (Kloosterman et al., 2010; Wolters et al., 2010). Furthermore, it was concluded that only one dominant allele has a major effect. Although *Chy2* has a major effect, there are apparently more genes contributing to the variation in flesh colour (Brown et al., 2006). Next to the yellow flesh colour, a darker colouring towards orange is also found. Allelic analysis of the zeaxanthin epoxidase (*Zep*) gene showed that in a set of different genotypes, all the accessions with orange flesh colour were homozygous for one specific *Zep* allele (Wolters et al., 2010). This finding was supported by the earlier observation that transformation of yellow fleshed

potatoes with *Zep* constructs showed an increase in the level of the carotenoid zeaxanthin (Römer et al., 2002).

Figure 2: Examples of white and yellow flesh colour in potato

Next to the colour traits, other obvious quality traits are the morphological traits like tuber size, shape, regularity of shape and eye-depth (see Figure 3 and 4 for examples). Tuber shape varies from long to compressed/round and is measured by the ratio between length and width. Long tubers can be used for French fries while round ones are preferred for crisps. However, unlike the colour traits and despite years of research the genetics of tuber morphology still remains unclear. The different traits are significantly correlated and some of the loci involved even seem to be genetically linked (Śliwka et al., 2008). For tuber shape a single locus on chromosome 10 was mapped with a dominant allele Ro conferring round tuber shape (Van Eck et al., 1994a). Other reports using populations with different genetic backgrounds mention QTLs on chromosomes 2, 5 and 11(Bradshaw et al., 2008), 2 and 11 (Śliwka et al., 2008) and 7, 12 and an unassigned linkage group (Sørensen, 2006). Thus, there are clearly more factors controlling this trait, probably depending on the genetic background, as different combinations of *Solanum* species were used to create the respective populations. One of the other morphological traits, eye depth, appears to be controlled by a single locus, which is closely linked with the Ro locus at chromosome 10 at a distance of 4 cM (Li et al., 2005b). This result was confirmed by another report (Śliwka et al., 2008), where two minor QTLs on chromosomes 3 and 5 were identified. The correlation between tuber shape and eye depth is shown by the linkage of deep eyes with round tubers (Li et al., 2005b). Little is known on regularity of shape. It depends on several components such as depths of indentations, various tuber defects and uniformity of shape and therefore will probably behave as a complex trait. Two QTLs on chromosomes 3 and 5 were reported for regularity of shape (Śliwka et al., 2008).

Figure 3: An overview of the variation in potato for tuber shape and eye depth.

Figure 4: An example of the variation present in potato for tuber size. The example is taken from the C x E population.

Besides a good appearance in terms of colour, size and shape, dormancy and shelf life are additional important quality traits for fresh market potatoes. The shelf life or storage capacity of potato tubers is controlled by many different processes. Respiration rate and dehydration of the tuber tissue during storage are determinants of tuber quality (Blenkinsop et al., 2003). Tuber dormancy, is another very important factor for ware potato shelf life. Improving storability of potato tubers through blocking sprouting has in the past been controlled by the use of chemicals like CIPC and DMN (Campbell et al., 2010). In Figure 5 an example of sprouting differences is shown. With regard to genetics, the main focus has been on unravelling the genetic processes behind dormancy. In two interspecific *Solanum* crosses a large number of QTLs was observed on chromosomes 2, 3, 4, 5, 7 and 8 (Freyre et al., 1994; Van den Berg et al., 1996), clearly indicating the quantitative nature of the trait. In potato, the genes involved in breaking dormancy and the following sprouting action, can be divided in several categories. A first group are the genes coding for homeotic proteins and transcription factors (Bachem et al., 2000; Faivre-Rampant et al., 2004). A second class of genes regulates hormone metabolism and hormone response. Abscisic acid and ethylene are mentioned as requirement for dormancy induction, while abscisic acid maintains dormancy and cytokinins are involved in loss of dormancy (Suttle,

2004). Supporting evidence for the role of abscisic acid was provided by co-localizing QTLs for tuber dormancy and abscisic acid content on chromosome 2 (Šimko et al., 1997; Śliwka et al., 2008; Van den Berg et al., 1996). Furthermore, microarray analysis showed a decrease in abscisic acid inducible transcripts particularly in the conserved BURP domain proteins, which include the RD22 class of proteins and in the storage protein patatin (Campbell et al., 2010). The third group of genes is involved in metabolism of reserve storage molecules (Horvath et al., 2002) (Agrimonti et al., 2007). Recently a fourth gene category was discovered, involved in DNA replication. In dormant meristems, cells are arrested in the G1/G0 phase of the cell cycle and re-entry into the G1 phase followed by DNA replication during the S phase enables bud outgrowth. Deoxyuridine triphosphatase (dUTPase) is essential for DNA replication and showed to be a marker for meristem reactivation in tuber buds predicting breaking of dormancy (Senning et al., 2010). All together dormancy is a very complex trait influenced by many different factors.

Figure 5: Differences in sprouting behaviour after breaking of dormancy in potato, the example is from offspring tubers from the segregating diploid C x E population.

There are two other traits with primary importance for consumption potatoes, namely taste and smell. Even though these may be important traits for consumers, research on taste and smell is relatively young. Furthermore, there is the complex nature of these traits, as over 250 different compounds have been identified in volatile fractions alone (Maga, 1994). This explains why the genetics behind these traits are still not well understood. Next to the volatiles, other soluble cellular constituents are important determining factors for the flavour as these define the basic taste parameters of sweet, sour, salty, bitter and umami (Halpern, 2000). A lot of the research has been dedicated to determining which other factors contribute to potato flavour, such as the

method of cooking, cultivar differences, effects of agronomic conditions and effects of storage (Blanda et al., 2010; Duckham et al., 2001; Oruna-Concha et al., 2002). The results on boiled potato volatiles include compounds derived from lipid degradation, the Maillard and Strecker reactions and include methional, aliphatic alcohols and aldehydes, thiols and sulfides and methoxypyrazines (reviewed in Taylor et al., 2007). Recently, boiled tuber volatiles produced by *S. tuberosum* cultivars have been compared with those from *S. phureja* cultivars (Shepherd et al., 2007). Flavour is one of the quality traits that differentiate *S. phureja* from *S. tuberosum* and the comparison is used to identify the key genes that underlie this trait. One of the volatiles that showed up in a gene expression experiment was α-copaene (Ducreux et al., 2008), which is an important aromatic compound in several food plants like lettuce and carrot (Nielsen and Poll, 2007). Low levels of α-copaene are present in *S. tuberosum*, but in *S. phureja* these levels are a 100-fold higher (Winfield et al., 2005). One other compound with clear relevance to taste is glutamate. Glutamate is a determinant of flavour in potato tubers because of its contribution to the umami taste as mentioned above (Morris et al., 2007). Genes encoding glutamate ammonia ligase and glutamine synthetase I, both part of the glutamate biosynthetic pathway, were expressed at higher levels in *S. phureja* cultivars. An overview of other differentiating genes can be found in Ducreux et al. (2008).

Nutritional quality of potato tubers

According to the WHO (2010) one third of the world child mortality is directly related to malnutrition (http://www.who.int/whosis/whostat). An effective treatment of this problem can be achieved by fortifying staple foods with extra nutritional factors. One of the most well known examples being the biotechnological enhancement of provitamin A in rice, resulting in the introduction of the so called golden rice (Paine et al., 2005). Even when malnutrition is not an issue, crop compositional quality is still of great importance from the perspective of human health (Demmig-Adams and Adams, 2002) and as GM crops are still not widely accepted for direct human consumption the focus on achieving high nutritional value through optimal use of the existing natural variation is evident.

In potato tubers the main dry matter component are carbohydrates, around 75% of the total dry matter. The largest portion of these carbohydrates is made up of starch (Camire et al., 2009). There is a large variation in starch content present in cultivated potato, 11,0-34,4% starch of fresh weight, and an even larger variation in the wild related species, 3,8-39,6% starch of fresh weight (Jansen et al., 2001). Although starch content has a strong genetic basis, a significant portion of the variation is due to the life cycle length of the potato plant. Late maturing cultivars

tend to have higher starch yield than early ones (Van Eck, 2007). Potato starch consists of two different polysaccharides, amylose and amylopectin on average in a one to four ratio. This ratio is important as it correlates with a number of different starch characteristics such as retro gradation of starch pastes (Visser et al., 1997b). It is this retro gradation that makes the starch to become more crystalline, leading to resistance to digestive enzymes. Retro-gradation, the breakdown by enzymes, is higher in higher-amylose starches. In general, starch in cooked potato is rapidly digestible, thanks to the relatively large amount of amylopectin (Lynch et al., 2007). A lot of research has already been put into elucidating the complete biosynthetic pathway of starch granule. Most of the enzymes involved in assembling starch granules are well known. The starch biosynthetic pathway works as follows. The substrate for the biosynthesis of starch polymers, ADP-glucose, is produced from glucose-6-P via glucose-1-P by phosphoglucomutase and ADP glucose pyrophosporylase (AGPase) respectively (Müller-Röber et al., 1992; Stark et al., 1992). ADP glucose is then used by the different starch synthase isoforms to construct the starch polymers. Starch synthases I, II and III (SSSI, SSSII and SSSIII) are mainly present in the soluble phase whereas the granule bound starch synthase I (GBSSI) is bound to the granule and catalyzes the synthesis of amylose (Kuipers et al., 1994a). Other enzymes as branching enzymes (SBE), debranching enzymes (DBE) and phosphorylating enzymes as α-glucan-diwaterkinase (GWD) complete the synthesis of starch granules (Hofvander et al., 2004; Ritte et al., 2002). For most of the genes above genome sequences are known, opening up the way for marker assisted breeding for starch content.

From a health point of view, protein, mineral, lipid, vitamin and other phytochemical content are equally important. However in contrast to the large amount of research on tuber starch content, the other nutritional compounds in potato have received less attention. Protein content is generally low in potato tubers, especially compared with other raw vegetable sources. Potato protein content ranges from 1-1,5% of tuber fresh weight in commercial varieties (Ortiz-Medina, 2007) to 0,3 to 1,2 % in specific research populations (Chapter 4, this thesis). Potato tuber proteins can be divided into three categories: patatins, protease inhibitors and other proteins (Pots et al., 1999). Patatin is the major protein present in potato tubers, accounting for up to 40% of the soluble protein (Prat et al., 1990). Two classes of patatin gene families have been observed (Pikaard et al., 1987). Class I transcripts were found to be tuber specific whereas the class II transcripts were observed in both tubers as well as roots (Prat et al., 1990). Patatin forms are encoded by highly similar gene copies within the patatin gene family, in total there are estimated to be 10 to 18 copies of patatin genes in each monoploid potato genome (Twell and Ooms, 1988). All the genes are suggested to map clustered on the same location on chromosome 8 of

the potato genome (Ganal et al., 1991). Several genes are already cloned and sequenced and transgenic approaches have been taken to knockdown patatin genes in order to get higher levels of other proteins, which are even more easier to purify (Hirschberg et al., 2001; Kim et al., 2008; Stupar et al., 2006). Further studies on the patatin gene family revealed that the genes are regulated by a DNA-binding protein acting as a transcription factor named storekeeper (Zourelidou et al., 2002). The other major class of tuber protein, protease inhibitors, have also been investigated thoroughly. This group of proteins can be divided into three classes based on molecular weight of the molecules. These proteins inhibit mainly proteases of the Kunitz type (Pots et al., 1999). The genes for Kunitz type inhibitors (KTI) form a gene family consisting of at least a dozen genes of which most map to a single locus named StKI on chromosome 3 of potato (Heibges et al., 2003; Odeny et al., 2010). The KTI genes have a proposed role in disease resistance and fall outside the scope of this review.

Vitamins are another important group of nutrients. Potato is not especially high in vitamin levels. Some of the B vitamins are observed in potato, although in low concentrations. Vitamin B1, B2, B3 and B6 were investigated in a series of cultivars. The observed variation ranged from 0,08-0,10, 0,022-0,36, 0,62-2,07 and 0,15-0,3 mg/100g fresh weight, respectively (Burlingame et al., 2009). However, the predominant vitamin in potatoes is vitamin C. The amount of vitamin C ranges in content between 84 to 145 mg per 100 g fresh weight depending on cultivar, agronomic practice and storage conditions (Augustin, 1975). Variation within parents of a potato breeding program was reported, with a four-fold difference in vitamin C content (Love et al., 2004). One of the beneficial attributes of vitamin C is improving the iron availability in potato tissue. Iron is a mineral that tends to be limiting in the human diet (Brown, 2008). Biochemically, vitamin C falls in the category of the antioxidants and this one property accounts for much of its health benefits. Other important antioxidants in potato tubers are carotenoids and anthocyanins. The genetics behind the carotenoid and anthocyanin biosynthesis are identical to the ones behind flesh colour and were discussed in the section about tuber flesh colour. Carotenoids are referred to as important for the human diet because of several health promoting activities. Most of these are antioxidant properties and are reported to be beneficial in preventing cancer, cardiac disease and eye diseases (Krinsky et al., 2004; Moeller et al., 2006).

From the other nutritional compounds, lipids are only a very small fraction of the tuber fresh weight amounting to approximately 0,15/150 grams fresh weight (Priestley, 2006), as such potatoes can basically be regarded as being fat free.

Potatoes are on the other hand an excellent source of minerals. 200gr Fresh weight of potato can provide roughly 26% of the U.S Dietary Reference Intake (DRI) of Cu, 17% of K, P and Fe and

5 to 13 % of Zn, Mg and Mn (White et al., 2009). Potatoes are a particularly good source of dietary iron. However, absorption of Fe^{2+} is inhibited by the presence of polyphenols and phytic acid. Potato is naturally low in these anti-nutrients, allowing a good uptake of iron from potato tubers (Brown, 2008). However, most of the research on potato mineral content focuses on the influence of agricultural measures rather than on the genetics underlying mineral content. In a relatively limited number of cultivars a broad range of iron content was observed, ranging from 18 to 65 µg/g dry weight. A less broad, but still promising range, was measured in the same cultivars for Zinc content ranging from 12,5 to 20 µg/g dry weight (Brown, 2008). A later publication indicated heritability scores for iron content to be between 0,64 and 0,76, with a clear environmental influence and genetic background specificity (Brown et al., 2010). The relatively high amounts of minerals and observed heritabilities combined with a broad observed range of amounts per mineral in different genetic material indicates that with breeding for mineral content a lot may still be gained.

Processing quality in potato

A third class of potato tuber quality traits, processing quality, has to do with the suitability of the tubers for processing. Two categories can be distinguished. First the preparation of French fries and chips or the processing of potatoes for direct food applications, like peeling and cutting potatoes for packaged ready to use potatoes for the consumer market. The second category is for pure industrial purposes like obtaining starch or fibres for a range of industrial applications.

While producing French fries or potato chips, reducing sugars (mainly glucose and fructose) react with the free amino acids in a non-enzymatic Maillard reaction leading to the production of brown- to black-pigmented products rendering the chips or fries unacceptable for consumers. These reducing sugars accumulate in the cold-storage of the potatoes prior to processing. The process of cold sweetening is a large problem for the potato processing industry. To illustrate this, in the US up to 20% of potatoes are rejected for processing because of high levels of reducing sugars (Sowokinos, 2004). Most of the genes involved in the processes underlying cold sweetening have been identified and molecularly characterized (Kumar et al., 2004; Sowokinos, 2001). During cold sweetening a change occurs in the carbon flux linking starch with sucrose, shifting the balance to an increase in sucrose synthesis (Isherwood, 1973). Furthermore, a large number of these genes have been cloned and mapped, starch phosphorylases, debranching enzyme, α-amylase (involved in starch degradation), sucrose phosphate synthase, sucrose synthases, invertases (involved in sucrose metabolism) and sucrose transporters (transport of sugars) (Chen et al., 2001; Menendez et al., 2002). Genetic mapping studies for chipping quality

and starch and sugar content seem to confirm the involvement of these genes (Li et al., 2008; Menendez et al., 2002). The step of hydrolyzing sucrose to glucose and fructose by invertase seems to be the most crucial for cold sweetening and its prevention. In an association mapping study specific alleles of the invertase loci *InvGE* and *InvGF* were correlated with better chipping quality (Li et al., 2005a). This was further confirmed when the potato vacuolar acid invertase gene *VInv* was silenced and potato chips were keeping a light colour even after long term storage at 4°C (Bhaskar et al., 2010) A side problem caused by the Maillard reaction during the production of chips or fries, is the creation of acrylamide, a neurotoxin and potential human carcinogen (Mottram et al., 2002; Stadler et al., 2002). Reducing sugars resulting from cold sweetening and asparagine are the two major substrates for acrylamide production in potato (Gökmen and Palazoğlu, 2008). Limiting the formation of reducing sugars is one solution of preventing acrylamide formation. Thus the associated genetics is the same as for cold sweetening as it is the same process that is causing the problem. Silencing of an invertase gene proved that this hypothesis is true for reducing acrylamide (Ye et al., 2010). The asparagine biosynthetic pathway is another route to finding a solution to the genetics of acrylamide formation. Asparagine synthetase is the last step in that pathway by converting aspartate into asparagine. Two cDNAs, StAs1 and StAs2, coding for this enzyme were used in a silencing construct. Transformed potato plants showed a 20 fold reduction in free asparagine in their tubers and the chips and fries made out of these tubers showed only 5% of the acrylamide observed in wild-type controls (Rommens et al., 2008b) The most effective way of limiting acrylamide content through a non-GMO approach would be to select for both lower reducing sugar content and asparagine levels, this approach was shown successful in a breeding population (Shepherd et al., 2010).

Another trait related to processing qualities of potato tubers is enzymatic discoloration. This process in which phenolic compounds are oxidized by the enzyme polyphenol oxidase (PPO), results in undesired dark pigments (melanins) (Friedman, 1997). This discoloration results in considerable economic losses for the food processing and retail industry. Prevention of enzymatic browning in food can be achieved by the use of sulfiting agents; however, in recent years doubt has risen about the safety of sulfites for human consumption, therefore making a breeding solution for this problem necessary. PPO has been reported to be one of the main causes for enzymatic discoloration (Vaughn et al., 1988). When PPO gene expression was silenced, the transformed lines showed a significantly reduced discoloration reaction (Bachem et al., 1994). PPO genes in potato form a genetically linked gene family of at least 6 genes which are differentially expressed, namely POTP1 and POTP2 (Hunt et al., 1993) and POT32, POT33,

POT41 and POT72 (Thygesen et al., 1995). POT32 is the major form expressed in tubers besides POT33 and POT72, whereas POTP1 and POTP2 are mainly expressed in leaves and flowers (Thygesen et al., 1995). Tissue specific expression of PPO genes was also reported in other crops like tomato (Newman et al., 1993). POT32 has been shown to be the cause of browning in *S. hjertingii*, where a truncated version of the gene prevents enzymatic discoloration (Culley et al., 2000). In *S. tuberosum* this gene was mapped to chromosome 8 and a clear correlation between allele combinations of POT32 and degree of discoloration was observed (Werij et al., 2007). In addition, analysis of POT32 gene expression in a subset of genotypes indicated a correlation between the level of gene expression and allele composition. Next to the PPO enzyme, the substrate content of potato tubers is also of importance for melanin formation. The main substrate consists of phenolic compounds. PPO oxidizes these phenolic compounds to melanins and 90% of the total phenolic content of potato tubers consists of chlorogenic acid. However, no correlation was observed between chlorogenic acid and discoloration (Friedman, 1997; Werij et al., 2007). Other studies on the possible substrate for PPO in the oxidation reactions did not result in clear indications to what substrate might be responsible (Corsini et al., 1992; Mondy and Munshi, 1993).

The last category of traits can be comprised under the header industrial applications. The vast majority of these traits are related to potato starch. Potato starch can be used for food applications, for instance as an additive to soups and sauces, as well as for non-food applications in industry, in papermaking paper coating, starch-based plastics, deep-drilling fluid control and pharmaceutical products (Li, 2008). For starch processing the most important characteristic is dry matter content. Dry matter content varies considerably over sites and seasons resulting in a variation in heritability (Tarn et al., 2006). Tuber starch content is directly affected by the potential of the starch biosynthetic pathway operating within the growing potato tubers. This pathway has been intensively studied in the last decades as already described in the section on cold sweetening. Specific QTL analysis on starch content has resulted in a large number of QTLs which at their turn can be connected with candidate genes. QTL regions on chromosome 1 include two starch phosphorylase genes, a gene coding for Rubisco on chromosome 2, a starch synthase gene on chromosome 3, a starch phosphorylase gene and a gene for a debranching enzyme on chromosome 9, a Rubisco activase gene and a gene coding for an invertase on chromosome 10, a sucrose transporter gene on chromosome 11 and a sucrose synthase gene on chromosome 12 (Chen et al., 2001). The function of some of these enzymes has been proven by a transgenic approach, including sucrose synthase (Zrenner et al., 1995), starch synthase (Kuipers et al., 1994b) and glucan branching enzyme (Kortstee et al., 1996).

Although we speak of starch related traits, the different applications of starch in food and non-food industries demand starch with different specifications. Wild type potato starch is a mix of the linear amylose and the branched amylopectin. Each of the two types has specific characteristics. Amylose has a high degree of polymerization due to its linear structure and is therefore suitable for applications in biodegradable plastics (Liu et al., 2003). For other applications, the polymer amylopectin is of interest, and amylose is regarded as an impurity as for example in industrial starch production. The creation of an amylose free potato solves the industrial problems of purifying the harvested potato starch. Natural mutants have been obtained that show amylose-free starch (Jacobsen et al., 1989), because of low viability a transgenic approach was used to obtain potato plants that had no amylose present (Visser et al., 1991). In this approach, the gene coding for granule-bound starch synthase (GBSS) was inhibited, GBSS being the enzyme responsible for amylose production.

An overview of all the QTLs and candidate genes mentioned in this review, combined with the results of this thesis, are shown in Table 2.

LG	QTL/Locus	Candidate Gene	Function	Quality trait	Reference
1	Sug1a / ts(l)	*AGPaseS(a)*	ADP-glucose pyrophosphorylase S	Cold Sweetening / Tuber starch content	Menendez et al, 2002; Chen et al, 2001
	Sug1b	-	-	Cold Sweetening	Menendez et al, 2002
	ts(s)	-	-	Tuber starch content	Chen et al, 2001
	ED-1	-	-	Ezymatic discoloration	Werij et al, 2007 (Chapter 2 of this thesis)
2	R	*dfr*	dihydroflavonol-4 reductase	Skin colour	Van Eck et al, 1993; Zhang et al, 2009
	Shape	-	-	Tuber shape	Bradshaw et al, 2008; Śliwka et al, 2008
	Dormancy	-	-	Dormancy	Freyre et al, 1994; Van den Berg et al, 1996
	Sug2a	-	-	Cold Sweetening	Menendez et al, 2002
	Sug2b	-	-	Cold Sweetening	Menendez et al, 2002
	ts(c)	*rbcS-c*	ribulose bisphosphate carboxylase	Tuber starch content	Chen et al, 2001
	CA-2	-	-	Citric acid content	Werij et al, 2007 (Chapter 2 of this thesis)
	SP02-2	*SSSIII*	Soluble starch synthase	Starch Phophorylation	Chapter 3 of this thesis
	SP03-2	*SSSII*	Soluble starch synthase	Starch Phophorylation	Chapter 3 of this thesis
	SGT02-2	*SSSII*	Soluble starch synthase	Starch Gelling Temperature	Chapter 3 of this thesis
	SGT03-2	*SSSII*	Soluble starch synthase	Starch Gelling Temperature	Chapter 3 of this thesis
	Am02-2	*SSSII*	Soluble starch synthase	Amylose Content	Chapter 3 of this thesis
	Am03-2	*SSSII*	Soluble starch synthase	Amylose Content	Chapter 3 of this thesis

LG	QTL/Locus	Candidate Gene	Function	Quality trait	Reference
3	Y	*chy2*	beta-carotene hdyroxylase	Yellow tuber flesh colour	Bonierbale et al, 1988; Thorup et al, 2000; Kloosterman et al, 2010
	Eye depth	-	-	Tuber eye depth	Śliwka et al, 2008
	Dormancy	-	-	Dormancy	Freyre et al, 1994; Van den Berg et al, 1996
	StKI	*kti*	Kunitz type inhibitors	Protein content	Heibges et al, 2003; Odeny et al, 2010
	Sug3a	-	-	Cold Sweetening	Menendez et al, 2002
	Sug3b	-	-	Cold Sweetening	Menendez et al, 2002
	Sug3c	-	-	Cold Sweetening	Menendez et al, 2002
	ts(g)	*Stp23 / rbcS-1*	starch degrading enzyme / ribulose bisphosphate carboxylase	Tuber starch content	Chen et al, 2001
	ts(h)	*SSSI*	Soluble starch synthase	Tuber starch content	Chen et al, 2001
	ED-3	-	-	Enzymatic Discoloration	Werij et al, 2007 (Chapter 2 of this thesis)
	Ch4C-3	*StPho1a*	Starch phosphorylase	Chipping color 4C	Chapter 3 of this thesis
	SGT03-3	*StPho1a*	Starch phosphorylase	Starch Gelling Temperature	Chapter 3 of this thesis
	SGS03-3	-	-	Average Granule Size	Chapter 3 of this thesis
	Am02-3	-	-	Amylose Content	Chapter 3 of this thesis
	Am03-3	-	-	Amylose Content	Chapter 3 of this thesis
4	Dormancy	-	-	Dormancy	Freyre et al, 1994; Van den Berg et al, 1996
	Sug4a	-	-	Cold Sweetening	Menendez et al, 2002
	Sug4b	-	-	Cold Sweetening	Menendez et al, 2002
	ts(i)	*Pk*	pyruvate kinase	Tuber starch content	Chen et al, 2001
	ts(k)	*SbeI / Pfp-α*	Starch branching enzyme / pyrophosphate fructose-6phosphate 1-phosphotransferase	Tuber starch content	Chen et al, 2001
5	Shape	-	-	Tuber shape	Bradshaw et al, 2008
	Eye depth	-	-	Tuber eye depth	Śliwka et al, 2008
	Dormancy	-	-	Dormancy	Freyre et al, 1994; Van den Berg et al, 1996
	Sug5a / ts(a)	*Sut2*	sucrose transporter	Cold Sweetening / Tuber starch content	Menendez et al, 2002; Chen et al, 2001
	Sug5b	-	-	Cold Sweetening	Menendez et al, 2002
	Ch4C-5	-	-	Chipping Color 4C	Chapter 3 of this thesis
	ChAR-5	-	-	Chipping color after recond.	Chapter 3 of this thesis
	CS(AH-4C)-5	*GWD*	Glucan water dikinase	Cold sweetening	Chapter 3 of this thesis
	CS(AH-AR)-5	*GWD*	Glucan water dikinase	Cold sweetening	Chapter 3 of this thesis
	SP02-5	*GWD*	Glucan water dikinase	Starch Phosphorylation	Chapter 3 of this thesis
	SP03-5	*GWD*	Glucan water dikinase	Starch Phosphorylation	Chapter 3 of this thesis
	Am02-5	*GWD*	Glucan water dikinase	Amylose Content	Chapter 3 of this thesis
	SGT02-5	-	-	Starch Gelling Temperature	Chapter 3 of this thesis
	SC98-5	-	-	Starch Content	Chapter 3 of this thesis
	SC99-5	-	-	Starch Content	Chapter 3 of this thesis

LG	QTL/Locus	Candidate Gene	Function	Quality trait	Reference
6	Sug6a	-	-	Cold Sweetening	Menendez et al, 2002
	Sug6b	-	-	Cold Sweetening	Menendez et al, 2002
	Sug6c	-	-	Cold Sweetening	Menendez et al, 2002
	ts(r)	-	-	Tuber starch content	Chen et al, 2001
	ts(d)	-	-	Tuber starch content	Chen et al, 2001
7	Dormancy	-	-	Dormancy	Freyre et al, 1994
	Sug7a	*Sps*	sucrose phospate synthase	Cold Sweetening	Menendez et al, 2002
	Sug7b	*Sus3*	sucrose synthase	Cold Sweetening	Menendez et al, 2002
	Sug7c	-	-	Cold Sweetening	Menendez et al, 2002
	Sug7d	-	-	Cold Sweetening	Menendez et al, 2002
	Sug7e	-	-	Cold Sweetening	Menendez et al, 2002
	ts(b)	*Pha2*	plasma membrane H+ ATPase 2	Tuber starch content	Chen et al, 2001
	Am03-7	-		Amylose Content	Chapter 3 of this thesis
8	ED-8	*POT32*	polyphenol oxidase	Enzymatic discoloration	Werij et al, 2007 (Chapter 2 of this thesis)
	Dormancy	-	-	Dormancy	Freyre et al, 1994; Van den Berg et al, 1996
		patatin	patatin	Protein content	Ganal et al, 1991
	Sug8a	-	-	Cold Sweetening	Menendez et al, 2002
	Sug8b	*AGPaseS(b)*	ADP-glucose pyrophosphorylase S	Cold Sweetening	Menendez et al, 2002
	Sub8c	-	-	Cold Sweetening	Menendez et al, 2002
	ts(m)	-	-	Tuber starch content	Chen et al, 2001
		GBSS	granule bound starch synthase	amylose content	Visser et al, 1991
	Tyr-8	-	-	Tyrosine content	Werij et al, 2007 (Chapter 2 of this thesis)
	Ch4C-8	-	-	Chipping color 4C	Chapter 3 of this thesis
	CS(AH-4C)-8	-	-	Cold Sweetening (AH-4C)	Chapter 3 of this thesis
	SGS02-8	-	-	Average Granule Size	Chapter 3 of this thesis
	SGS03-8	-	-	Average Granule Size	Chapter 3 of this thesis
	SC98-8	-	-	Starch Content	Chapter 3 of this thesis
	SC99-8	-	-	Starch Content	Chapter 3 of this thesis
9	Sug9a	*Inv-ap(b)*	apoplastic invertase	Cold Sweetening	Menendez et al, 2002
	ts(o)	-	-	Tuber starch content	Chen et al, 2001
		InvGE/GF	vacuolar invertase	Cold Sweetening	Li et al, 2005
	SP02-9	-	-	Starch Phosphorylation	Chapter 3 of this thesis
	SP03-9	*StPho2*	Starch phosphorylase	Starch Phosphorylation	Chapter 3 of this thesis
	ChAH-9	*StPho2*	Starch phosphorylase	Chipping Color after harvest	Chapter 3 of this thesis

LG	QTL/Locus	Candidate Gene	Function	Quality trait	Reference
9	SGS02-9	*StPho2*	Starch phosphorylase	Average Granule Size	Chapter 3 of this thesis
	SC98-9	-	-	Starch Content	Chapter 3 of this thesis
	SC99-9	-	-	Starch Content	Chapter 3 of this thesis
10	D	*R2R3 MYB*	transcription factor	Skin colour	Van Eck et al, 1993; Jung et al, 2009
	RO	-	-	Round tuber shape	Van Eck et al, 1994a
	Eyd	-	-	Tuber eye depth	Li et al, 2005b;
	Sug10a	*Inv-ap(a)*	apoplastic invertase	Cold Sweetening	Menendez et al, 2002
	ts(f)	*Rca*	ribulose bisphosphate carboxylase activase	Tuber starch content	Chen et al, 2001
	ChAH-10	*StLin8*	invertase	Chipping Color after harvest	Chapter 3 of this thesis
	Ch4C-10	*StLin8*	invertase	Chipping Color 4C	Chapter 3 of this thesis
	ChAR-10	-	-	Chipping Color after recond.	Chapter 3 of this thesis
	Am02-10	-	-	Amylose Content	Chapter 3 of this thesis
	SC98-10	-	-	Starch Content	Chapter 3 of this thesis
	SC99-10	-	-	Starch Content	Chapter 3 of this thesis
11	P	*f3'5'h*	flavonoid 3',5'-hydroxylase	Skin colour	Van Eck et al, 1993; Jung et al, 2005
	Shape	-	-	Tuber shape	Bradshaw et al, 2008; Śliwka et al, 2008
	Sug11a	*Sut1*	sucrose transporter	Cold Sweetening	Menendez et al, 2002
	ts(n)	*DbeI*	de-branching enzyme	Tuber starch content	Chen et al, 2001
12	Sug12a	-	-	Cold Sweetening	Menendez et al, 2002
	ts(e)	*Ppa1(a)*	soluble inorganic pyrophospatase	Tuber starch content	Chen et al, 2001
	ts(p)	*Sus4*	Sucrose synthase	Tuber starch content	Chen et al, 2001
	SC98-12	-	-	Starch Content	Chapter 3 of this thesis
	SC99-12	-	-	Starch Content	Chapter 3 of this thesis
Unknown		*zep*	zeaxanthin epoxidase	Orange tuber flesh colour	Wolters et al, 2010
Unknown		*dUTPase*	deoxyuridine triphosphatase	Dormancy	Senning et al, 2010
Unknown		*nsLTP*	non-specific lipid-transfer proteins	Dormancy	Horvath et al, 2002
Unknown		*G1-1*	unknown	Dormancy	Agrimonti et al, 2007
Unknown		*storekeeper*	DNA-binding protein	Protein content	Zourlidou et al, 2002
Unknown		*StAs-1 / StAs-2*	asparagine synthase	Acrylamide formation	Rommens et al, 2008b

Table 2: QTL loci and candidate genes, per chromosome, linked to quality traits in potato. This overview is based on the literature discussed in this review and results from this thesis and includes also gene function and relevant reference. Due to an insufficient amount of common markers between the different reports, map positions in cM of QTLs and candidate genes are not shown.

The genetic principles of quality

What becomes clear from the analysis of the traits that make up the quality aspect of potato tubers is that in a many cases the underlying genetics is very complex. The large number of QTL studies discussed in this chapter, highlight this complexity. Of all the traits breeders are working on, breeding for quality traits is often the most complex, more complex for instance than resistance breeding. Breeding for resistance in potato is, next to the few quantitative resistances, based on the gene-for-gene model (Flor, 1971). For every resistance gene in the plant there is a corresponding avirulence gene in the pathogen, limiting the genetics to a single gene. For quality traits the resulting phenotype is often based on the interactions of a multitude of genes acting together in complete metabolic pathways, like in the case of cold sweetening where both the starch biosynthetic as well as the starch degradation pathway play a role in the observed variation for cold sweetening. Another example is tuber flesh colour where anthocyanin and carotenoid pathways determine the final flesh colour (Brown et al., 2007). Within these biosynthetic pathways there is a certain level of redundancy where steps can be influenced by several enzymes, thereby only showing a clear effect when all of the genes involved are targeted. Eliminating only one gene, for instance by transgenic silencing might not have the desired effect as its function is taken over by other genes. The only way of influencing the output of complete biosynthetic pathways is by targeting key steps in the routes. An example here is the successful silencing of the GBSS gene in potato resulting in the amylose-free phenotype (Visser et al., 1991). Influencing a biosynthetic route via genetic modification is one way, using the available genetic variation by selecting for the most desired allele of the gene coding for a key enzyme is a way with a broader acceptance and therefore broader applicability. For quality traits it often comes down to the presence of a specific allele in order to get the desired phenotype. Clear examples of this principle are shown for POT32 involved in enzymatic discolouration (Werij et al., 2007) where homozygosity for one particular allele caused the highest degree of discoloration. An other example is flesh colour where out of eleven different alleles of the *Chy2* gene, only one turned out to be responsible for the difference between yellow or orange flesh colour (Wolters et al., 2010). This dependence on a specific allele to get the desired phenotype is key factor in breeding for quality traits.

Breeding for quality requires a basic understanding of the pathways underlying the trait of interest. Furthermore, identifying the genes involved and how they function and are regulated within the biosynthetic routes is an important prerequisite to understanding the biology of phenotype expression. As last step in this process, it is crucial to identify the existing allelic

variation of these key genes and use the allele that delivers the desired phenotype in breeding programs.

Breeding for quality

With the emphasis of a lot of breeding programs on increasing yield performance with every new variety on the market and a lot of focus on pathogen resistance as an insurance of reaching the yield potential, breeding for quality is thus a second priority. This lack of interest in quality is even more profound when breeding for yield has a direct negative effect on quality, for instance in tomato were breeding for yield has led to loss of flavour quality (Goff and Klee, 2006). This specific conflict of interests is referred to as the breeders dilemma (Morris and Sands, 2006). It will be obvious that a clear view of how to handle quality traits in a breeding program is crucial. One of the first steps is assessing the available genetic variation for a trait, within cultivated species but also in wild relatives. Especially in potato there is a large amount of variation present in the region of origin in South America, where farmers still use several species of potato and where still over 5000 potato varieties are grown ranging from diploid to tetraploid species (Lutaladio and Castaidi, 2009). In those species and cultivars a large source of variation is present that can exceed the variation present in modern potato varieties. Introgression of traits from related species has been extensively used for resistance breeding and it has proven its value as an important breeders tool. A clear example are the R3 resistance genes conferring resistance to *P. infestans* from *S. demissum* which were introgressed into *S. tuberosum* (Huang et al., 2004). A quality trait for which this principle is likely to work is flesh colour, where in the group referred to as 'Papa Amarilla', composed of the diploid groups *S. phureja, S. stenotomum* and *S. goniocalyx*, high levels of carotenoids are present. Levels exceeding those in modern tetraploid cultivars (Brown et al., 2007). Variation in related species is also found for dormancy, where in *S. jamessii* a dormancy period of eight years is observed (Bamberg, 2010). From these examples it may be clear that also for quality traits introgression of variation from related 'wild' species can be very beneficial for the variety development.

Establishing variation is the first step, understanding how the variation works is the next. Unravelling the genetics under all the variation is classically done by setting up a population using two extreme phenotypes as parents, phenotyping this population and performing a QTL analysis in order to understand the genetic locations influencing the trait of interest. In potato, genetic studies have predominantly been performed using a diploid population (Ganal et al., 1991; Menendez et al., 2002; Werij et al., 2007), as the genetic principles are more straightforward than in a tetraploid population. Recently however, tetraploid populations have

also been used (Bradshaw et al., 2008). In order to circumvent the necessity of creating a population with the risk of limiting the genetic variation for the trait of interest, a new method arising from the human genetics has recently been used. Association mapping has been performed in potato using a set 221 tetraploid cultivars looking for the genetic components of several quality traits (D'Hoop et al., 2008). The greatest advantage of this method is the fact that tetraploid varieties can be used, maintaining the proper genetic background and circumventing linkage drag of undesired genetic material. Variety or pedigree databases are very useful tools to use as a data source for association analysis. Several databases have been created that are publicly available. Examples are the potato pedigree database (http://www.plantbreeding.wur.nl/potatopedigree/), the European cultivated potato database (http://www.europotato.org/menu.php) and the British potato variety database (http://varieties.potato.org.uk/menu.php). When genetic regions are identified that influence your trait of interest, linking QTLs with genes and functions is the next and usually challenging step. Traditionally this depends heavily on prior knowledge of similar traits, pathways and genetics in the crop of interest or related crops. Other resources encompass EST databases and cDNA libraries with sequences that can be used to develop molecular markers for candidate genes that can then be localised on genetic maps. Subsequent co-localizing QTLs and candidate genes form literature-based indications of the relation between a gene and its function. Developing genetic markers, either in the close proximity of the gene or directly based on the gene sequence, enables the breeder to indirectly steer variation and select new varieties with the desired phenotype.

Recent technological developments have fundamentally changed the way genetic research and eventually breeding is performed at this moment and they will have an even larger impact in the very near future. Since the publication of the first genome sequences (genomics) at the beginning of this century clear progress has been made towards defining which genes are induced or repressed (transcriptomics), what proteins are produced (proteomics) and the way metabolic processes are influenced by these proteins (metabolomics). All this knowledge comes together under the term ~omics and offers a valuable tool for researchers and breeders to use and it opens up a whole new scope of possibilities in understanding trait biology and applying precision breeding for new varieties.

In the field of genomics the focus is on the study of genome sequences and the fine-scale genetic mapping efforts. For potato the resources have significantly grown over the years, including Expressed Sequence Tags (EST's) (Flinn et al., 2005; Ronning et al., 2003), bacterial artificial chromosome (BAC) clone libraries (Song et al., 2000), a dense genetic map (Van Os et al., 2006), a POCI 44k microarray (Kloosterman et al., 2008) and recently the genome sequence of

potato (PGSC, 2011). Moreover, the underlying genetics of quality traits can be delivered by the use of transcriptomics tools, through the analysis of RNA molecules in the broadest sense. cDNA libraries for potato are available (Crookshanks et al., 2001) and can be used to develop primer sequences for candidate gene analysis. Microarray platforms can be used to study the expression patterns of genes (Kloosterman et al., 2005). The science of metabolomics can help to solve the gaps in the knowledge that we have of the biosynthetic pathways and regulatory networks underlying the phenotypic quality traits that are of importance to breeders. Elucidation of these pathways can lead to the identification of genes encoding the biosynthetic enzymes. As such, each of these fields of research offers promising results, however integrating the different areas of expertise provide powerful tools for gene discovery and understanding of complex genetics.

Objectives of this thesis

The research described in this thesis is embedded within the Centre for BioSystems Genomics (CBSG) based in Wageningen, the Netherlands. The centre provides a research environment for several different crops and technologies, amongst which is potato. Within the potato cluster the focus is on *Phytophthora infestans* research and research on potato quality traits. This interest in potato quality traits is expressed in the research published in this thesis.

The objective of this thesis is gaining understanding of the genetic principles underlying a series of different potato quality traits and the development of molecular markers linked to the traits of interest. Molecular markers can be used in breeding programs with the aim of producing potato varieties with improved quality. The research was focussed on quantitative trait locus (QTL) and candidate gene analysis, supplemented by transcriptome and metabolome analysis.

Chapter 2 describes the QTL mapping of enzymatic discoloration (ED) of potato tubers and the supposed substrates chlorogenic acid and tyrosine. Mapping of the candidate gene POT32 coincided with QTLs for ED. POT32 showed allele specific expression patterns that could be linked to phenotypic classes of ED.

Chapter 3 reports the QTL analysis in combination with a candidate approach of potato starch related traits. In particular chipping colour, cold induced sweetening, starch content, starch granule size, starch gelling temperature, starch enthalpy, amylose content and degree of starch phosphorylation.

The genetic analysis of the metabolic variation present in tubers of the diploid potato population C x E is presented in Chapter 4. The mQTL results were compared with QTL results for tuber

flesh colour (raw and after-cooking) and total tuber protein content in order to identify metabolites of influence to the investigated traits.

Finally the results of this thesis are discussed with respect to the genetics of potato quality traits and marker assisted breeding with the aim to improve tuber quality.

Chapter 2

Unravelling enzymatic discoloration in potato through a combined approach of candidate genes, QTL -and expression analysis

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Abstract

Enzymatic discoloration (ED) of potato tubers was investigated in an attempt to unravel the underlying genetic factors. Both enzyme and substrate concentration have been reported to influence the degree of discoloration and as such this trait can be regarded as polygenic. The diploid mapping population C x E, consisting of 249 individuals, was assayed for the degree of ED and levels of chlorogenic acid and tyrosine. Using this data, Quantitative Trait Locus (QTL) analysis was performed. Three QTL's for ED have been found on parental chromosomes C3, C8, E1 and E8. For chlorogenic acid a QTL has been identified on C2 and for tyrosine levels, a QTL has been detected on C8. None of the QTLs overlap, indicating the absence of genetic correlations between these components underlying ED, in contrast to earlier reports in literature. An obvious candidate gene for the QTL for ED on Chromosome 8 is polyphenol oxidase (PPO) which was previously mapped on chromosome 8. With gene specific primers for PPO gene POT32 a CAPS marker was developed. Three different alleles (POT32-1, -2 and -3) could be discriminated. The segregating POT32 alleles were used to map the POT32 CAPS marker and QTL analysis was redone, showing that POT32 coincides with the QTL peak. A clear correlation between allele combinations and degree of discoloration was observed. In addition, analysis of POT32 gene expression in a subset of genotypes indicated a correlation between the level of gene expression and allele composition. On average, genotypes having two copies of allele 1 had both the highest degree of discoloration as well as the highest level of POT32 gene expression.

Introduction

Enzymatic discoloration in potato tubers is the process in which phenolic compounds are oxidized by the enzyme polyphenol oxidase (PPO) to quinones after which the quinones are transformed to dark pigments (melanins) (Friedman, 1997). This discoloration results in considerable economic losses for the food processing and retail industry. Prevention of enzymatic browning in food can be achieved by the use of sulfiting agents; however, in recent years doubt has risen about the safety of sulfites for human consumption. An environmentally and nutritionally safe approach would be to breed new potato cultivars which do not display enzymatic discoloration. Breeding for enzymatic discoloration resistant potatoes first requires a thorough understanding of the genetic factors underlying the trait. Until now, research has been done to dissect the underlying mechanisms of the trait. In general two approaches were taken, either focussing on the enzyme PPO or on the substrate of the enzymatic reaction. From this research, PPO has been reported to be one of the main causes for enzymatic discoloration (Vaughn et al., 1988). When the PPO gene expression was silenced, the transformed lines showed a significantly reduced discoloration reaction (Bachem et al., 1994). PPO genes in potato comprise a genetically linked gene family of at least 6 genes, namely POTP1 and POTP2 (Hunt et al., 1993) and POT32, POT33, POT41 and POT72 (Thygesen et al., 1995). The genes are differentially expressed, with POT32 being the major form expressed in tubers besides POT33 and POT72. POTP1 and POTP2 are mainly expressed in leaves and flowers (Thygesen et al., 1995). This tissue specific expression of PPO genes was also demonstrated by (Newman et al., 1993) in tomato. The tomato PPO genes share greater sequence similarity with their orthologs in potato than with their paralogous PPO genes in tomato. POT32 has been shown to be the cause of browning in *Solanum hjertingii*, where a truncated version of the gene prevents enzymatic discoloration (Culley et al., 2000).

Besides the enzymatic aspects of PPO on enzymatic discoloration, there is also a substrate aspect to the trait. The main substrate consists of phenolic compounds. As mentioned, PPO oxidizes phenolic compounds to melanins and 90% of the total phenolic content of potato tubers consists of chlorogenic acid although no correlation was observed between chlorogenic acid and discoloration (Friedman, 1997). A high correlation between tyrosine content and the results of the abrasive peel test, comparable with enzymatic discoloration, was detected (Corsini et al., 1992). Another important determinant is the partitioning of tyrosine between tuber protein and the free amino acid pool, where high free amino acid levels corresponded with a high level of discoloration (Corsini et al., 1992). However, other research reported that in spite of an observed

high correlation between free tyrosine and discoloration, the amount of tyrosine does not seem to be the determining factor for enzymatic discoloration (Mondy and Munshi, 1993).
Based on the hypothesis that multiple factors, at least enzyme and substrate, are involved in determining enzymatic discoloration we can regard the trait enzymatic discoloration as being a quantitative trait. Quantitative Trait Locus (QTL) analysis (Lander and Botstein, 1989), making use of a diploid potato population and its genetic map, can dissect the underlying genetic factors responsible for the variation observed in this trait. In this paper we present a combined approach including QTL mapping for enzymatic discoloration and the subsequent substrates, candidate gene mapping and expression assays for polyphenol oxidase in order to unravel the complex trait of enzymatic discoloration.

Materials and Methods

Plant Material

The diploid potato mapping population C x E, descending from the backcross between C (US-W5337.3) (Hannemann and Peloquin, 1967) and E (77.2102.37) (Jacobsen, 1980), was used. In the current research 249 full sib descendants were selected for which the morphological observations (enzymatic discoloration) were done and an AFLP based linkage map was created (Celis-Gamboa, 2002). All clones were grown in multi year repeats in the field (Wageningen, the Netherlands) during the normal potato growing season in the Netherlands (April-September).

Measuring the degree of enzymatic discoloration

The field grown potato tubers were harvested and analyzed directly after harvest. One random tuber per genotype was peeled, grated and exposed to air in a small Petri dish at room temperature. The degree of discoloration was measured after 30 minutes and after three hours. The measurements for each genotype were done in 2 replicates and per replicate 3 tubers were tested. Based on the observed discoloration, the F1 population was visually classified on an ordinal scale in 9 different classes (0 till 8), ranging from no discoloration at all to completely brown/black. The phenotypic analysis was performed in two consecutive years (1998 and 1999).

Measuring tyrosine and chlorogenic acid content

Tyrosine and chlorogenic acid content were measured in a subset of 100 randomly selected clones of the C x E population. The chlorogenic acid content (levels of 3-, 4- and 5-caffeoyl quinic acid separate) was determined for the harvest of 2002, the tyrosine levels were determined for the harvest of two consecutive years, 2002 and 2003. Directly after harvest 500 g of tubers

were selected per clone and peeled, sliced and frozen in liquid nitrogen. Then the frozen slices were grinded to a very fine powder in liquid nitrogen and stored before analysis at -80º C. The analysis of chlorogenic acid levels was done using LCMS based methods according to (Moco et al., 2006). All samples were measured once and both parent lines were measured six times, in between the progeny samples, to control the stability of the assay. For the tyrosine analysis, tissue (0.5-1.0 g) was homogenized with a mortar and pestle in 2 ml 50 mM Pi-buffer (pH 7.0) containing 1 mM dithiothreitol. Nor-leucine was added as an internal standard. Free amino acids were partly purified by extraction with 5 ml of a water:chloroform:methanol mixture (3:5:12). The water phase was collected and the organic phase was re-extracted twice. After concentration by lyophilisation to 3 ml, a 20 µl sample was analyzed by HPLC using a cation-exchange column with post-column ninhydrine derivatisation of the amino acids detected at 570 and 440 nm (BIOCHROM 20, Amersham Pharmacia biotech).

QTL mapping

The phenotypic data were tested for normality and, when not normally distributed, transformed (square root). The parental maps used in this experiment are obtained from (Celis-Gamboa, 2002) with minor modifications. The paternal map consists of 180 dominantly scored AFLP markers grouped together in twelve linkage groups representing the twelve chromosomes. The maternal map contains 141 AFLP markers subsequently grouped together representing the twelve chromosomes. QTL analysis was performed using the software package MapQTL® version 5.0 (Van Ooijen, 2004). Although the phenotypic data consists of values on an ordinal scale, QTL analysis was done using the interval mapping method developed by Lander and Botstein (1989). The results of the interval mapping were compared to the results of the Kruskal Wallis test and no differences between the results of the two analyses were observed. Detection of a QTL was done using a LOD threshold calculated with the permutation test option provided in MapQTL® (Van Ooijen, 2004).

CAPS marker development

Initially, two forward and reverse primer sets were developed based on mRNA sequence (GB accession number *U22921*) obtained from NCBI (http://www.ncbi.nlm.nih.gov/). Primers were synthesized by Biolegio BV (Malden, The Netherlands). Primer sets POT32PS1 and POT32PS2 were designed to specifically amplify POT32 sequence through the identification of POT32 specific regions based on alignment of different PPO sequences (POT32 GB U22921, POT33 GB U22922, POT72 GB22923, PPO-P1 and PPO-P2 (Hunt et al. 1993)). POT32PS1 forward

primer 5'-CTTCTTCCTCCACTTCTTT -3' and reverse primer GGGCAAAGTTGAACCT -3' and POT32PS2 forward primer 5'-CCAAAACGTTGAAACAAA -3' and reverse primer 5'-CTCTTGTGCATTTTTCTCT -3'. PCR reactions were performed using 1µl of DNA (5 µg/µl), 1.0 µl of each primer (1 µg/ml), 1µl of dNTPs (5mM), 5 µl of PCR buffer and 0.1µl of Super Taq polymerase (5 U/ml) (Sphaero Q, Leiden, The Netherlands) in a total volume of 20 µl. The PCR reactions for both sets consisted of a start of 120 sec at 94ºC and followed by 35 cycles of 94ºC for 30 sec, 52ºC for 30 sec, 72ºC for 60 sec and finished by 72ºC for 300 sec. The PCR reactions were performed on PE-9600 thermocycler machines from Perkin-Elmer Applied Biosystems Inc (Foster City, CA, USA). PCR results were analyzed on 1% agarose gel. PCR fragments were directly cloned into the pGEM®-T Easy vector system (Promega Corporation, Madison, WI, USA) using the standard protocol. Plasmid DNA samples, including inserted fragments, were send to Baseclear BV (Leiden, The Netherlands) for sequencing. Sequences were analyzed using the Vector NTI software package (Informax Inc, Bethesda, Maryland, USA) The Vector NTI software was also used to identify candidate restriction enzymes with a restriction site polymorphism between the alleles. The predicted restriction site polymorphisms were verified on POT32PS1 PCR fragments amplified from genomic DNA of the parental clones.

Expression analysis

A selection of genotypes (28 clones) with an extreme phenotype for either strong or weak enzymatic discoloration was grown in pots in the greenhouse. Similar sized tubers of a single plant, representing a similar developmental stage having had at least a four week period of tuber growth, were selected for expression studies. RNA was isolated as described by Bachem et al. (1996). Relative Expression levels of POT32 were determined by real-time qRT-PCR on a Perkin Elmer Abi Prism 7700 Sequence detector (Perkin Elmer, Nieuwerkerk, The Netherlands) following the protocol described by Kloosterman et al. (2005). Potato ubiquitin primers (*ubi3*) were used as a control. Relative quantification of the target RNA expression level and standard deviation was performed using the comparative Ct method according to the User Bulletin #2 (ABI Prism 7700 Sequence Detection System, December 1997, Applied Biosystems). The primer sequences for the genes studied are as follows: POT32 (GB accession number *U22921*) forward primer 5'-AATGCTCCATGTCCTCGGAT -3', reverse primer 5'-AACCCCGAGATCATAAGGCC -3' and *ubi3* (GB accession number *L22576*) forward primer 5'- TTCCGACACCATCGACAATGT-3', reverse primer 5'-CGACCATCCTCAAGCTGCTT-3'.

Results

Phenotypic analysis

Enzymatic discoloration values of the population represent the means of all replicates, over years and repetitions. The values showed a large variation, ranging from 0 to 8, several clones showed no discoloration at all, even after 3 hours, while other genotypes developed severe discoloration (Figure 1A). Enzymatic discoloration shows a transgressive segregation pattern in which both parental clones have intermediate phenotypes. The broad sense heritability's were estimated as 0.84 for discoloration after 30 minutes and 0.82 for discoloration after 3 hours.

Since tyrosine and chlorogenic acid (as the main polyphenolic compound) are the principal substrates for enzymatic browning, the levels of these compounds were determined for each potato clone. The results of these measurements are shown as averages over two years per clone for tyrosine in figure 1B and for chlorogenic acid as square root transformed values (to compensate for the absence of normal distribution) of one year in figure 1C. The smallest amount of tyrosine present was 0.232 µmol per gram fresh tuber flesh and the highest amount was 2.143 µmol per gram, a more than nine fold increase in tyrosine content between the two genotypes. For this trait the heritability was estimated to be 0.88. The three different chlorogenic acid forms (3-, 4- and 5-caffeoyl quinic acid) investigated, showed a variation of 10 to 50 fold changes in amount between clones. The correlation coefficient between the degree of enzymatic discoloration and tyrosine levels was 0.20 (with p-value is 0.05) and between enzymatic browning and chlorogenic acid levels the correlation was 0.13 (with p-value is 0.20). This indicates that based on the data obtained from this population, we can conclude that there is no relationship between substrate levels and the degree of enzymatic discoloration.

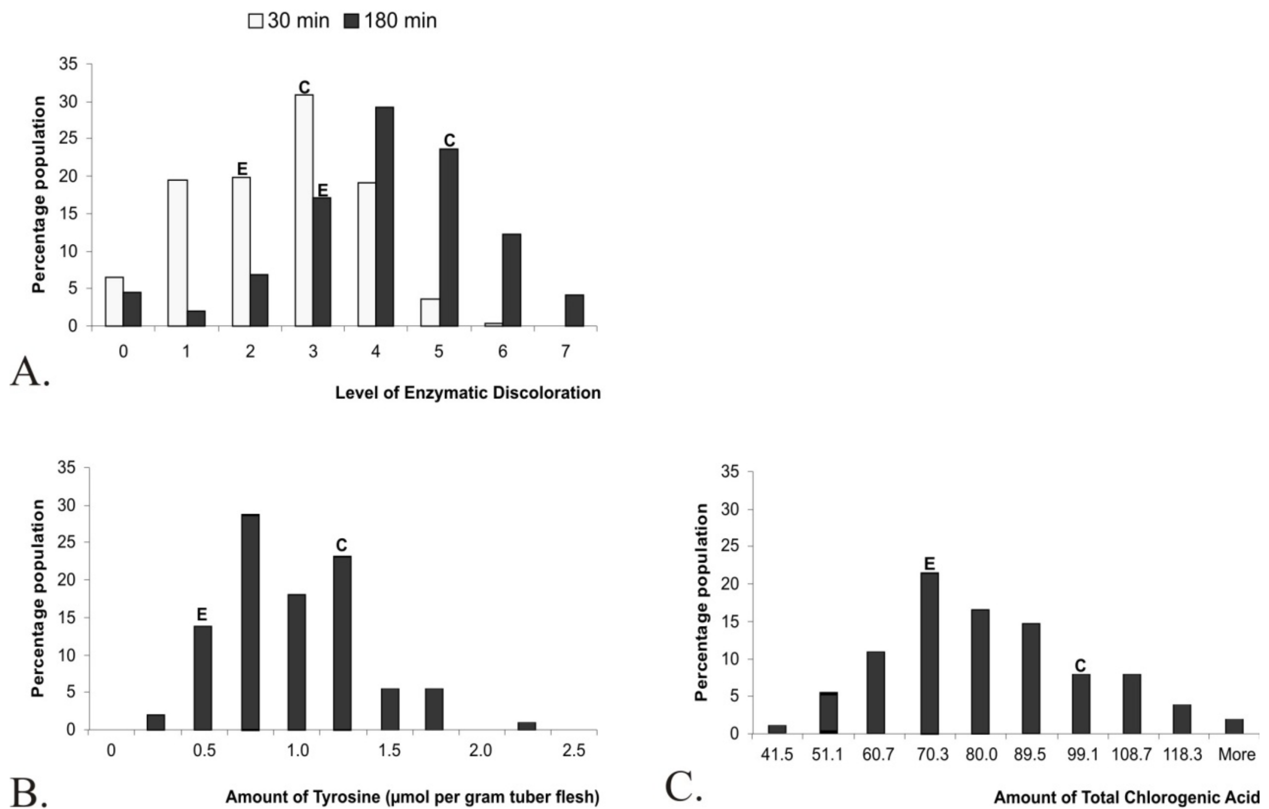

Figure 1: Distribution of the trait values in the C x E offspring. Letters C and E above the graphs indicate parental values. A. Degree of enzymatic discoloration on a scale of 0 (no discoloration) to 8 (dark brown/black). B. Tyrosine content (µmol per gram tuber flesh). C. Chlorogenic acid (relative scale).

QTL mapping

In order to identify the chromosomal regions contributing to the phenotype of enzymatic discoloration, tyrosine levels and chlorogenic acid levels, a QTL analysis was performed. QTL's were identified based on a genome wide significant threshold obtained by performing a permutation test on the data set.

For both time points at which discoloration was determined, QTL's were identified on both the C and E parental maps. The QTL's of the different time points coincide precisely. However, since the QTL effects at the 30 minutes time point are significantly larger than those at 3 hours, which do not all reach the LOD threshold, only the QTL's for the 30 minutes time point are shown in Figure 2A.

In total, four QTL's were identified affecting the degree of enzymatic discoloration, two on the maternal map (ED-3 on chromosome C3 and ED-8a on C8) and two on the E map (ED-1 on chromosome E1 and ED-8b on E8). The QTL's on chromosome 8 of both the parental maps are homologous to each other, both co-localizing with the markers for POT32. QTL ED-8a explains the largest part (21%) of the total variance for this trait. The QTL's on C3, E1 and E8 have

explained variances of 20%, 12% and 16% respectively, although both QTL's on chromosome 8 explain, partly, the same variance twice. The candidate gene POT32 (see below), specifically allele 1, shows the highest association with the QTL on chromosomes 8 of both the parental maps in the non-parametric Kruskal-Wallis test (K* = 33,227 with 1 df and p-value < 0.0001).

QTL analysis for chlorogenic acid content was performed on all the three forms (3-, 4-, 5-caffeoyl quinic acid) independently and on total chlorogenic acid content. Only the analysis for 5-caffeoyl quinic acid gave a significant result above the LOD thresh hold. One single QTL (CA-2) was detected on chromosome C2 (Figure 2B). The QTL has a LOD score of 3.80 and explains 21% of the total variance. For the other substrate, tyrosine, QTL analysis was performed using the averaged data over the two harvest years. The analysis resulted in a single significant QTL at chromosome C8, Tyr-8 (Figure 2C), with a LOD score of 5.76 and an explained variance of 36%.

As can be seen from Figure 2, the QTL's for enzymatic discoloration and for both the substrates do not co-localise. Both enzymatic discoloration and tyrosine content do show a QTL on chromosome C8. However, the QTL's are more than 20 cM apart, without an overlap in the QTL intervals, thus indicating that both QTL's are not related. Although the QTL analysis of enzymatic discoloration results in 3 different QTL's and the substrate based components result in 2 QTL's, none of these QTL's are overlapping. Against our expectations we can not prove a clear correlation between enzymatic discoloration and substrate levels.

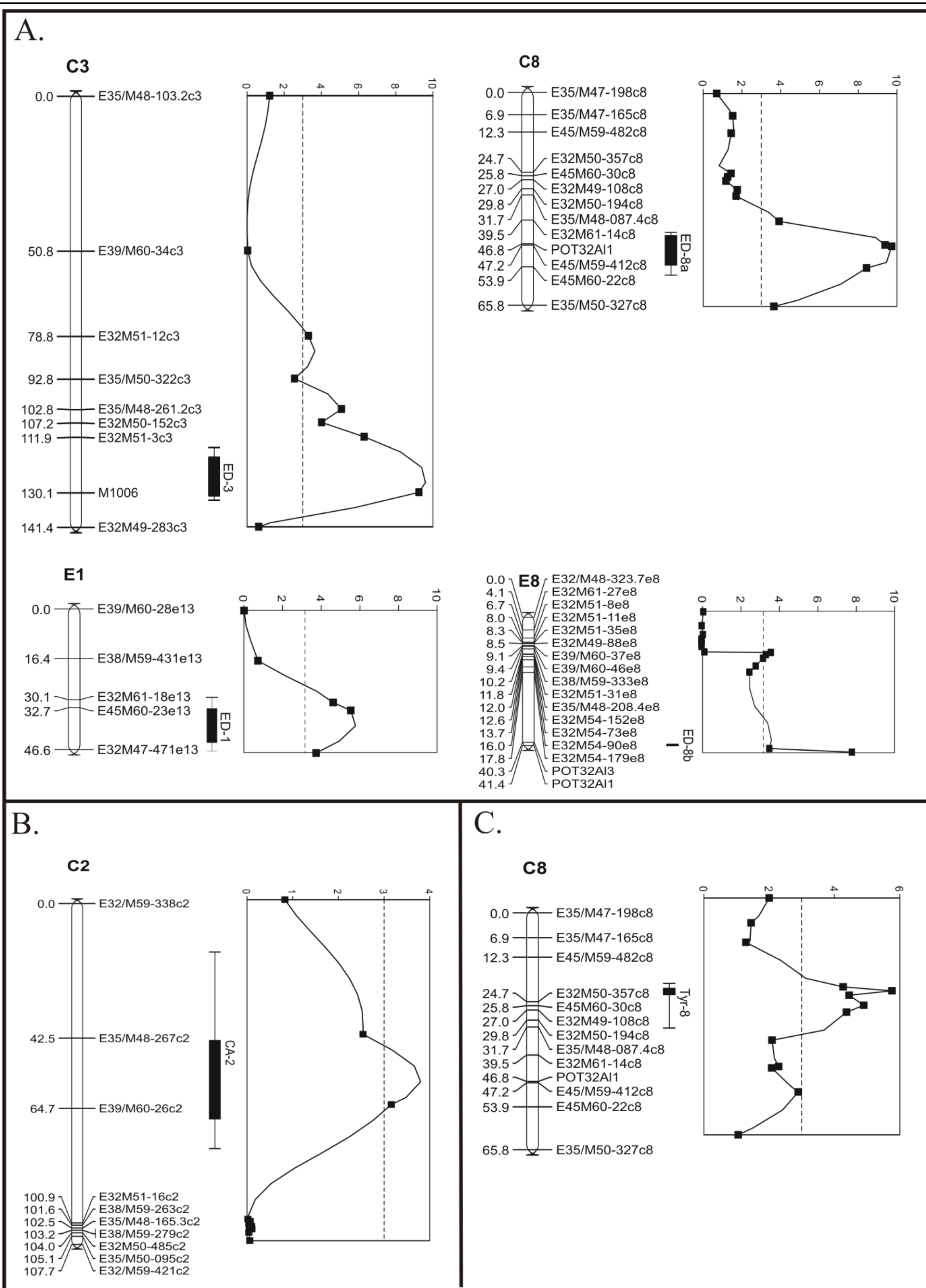

Figure 2: A. LOD profiles of the interval mapping analysis for the trait enzymatic discoloration. Shown are the observed QTL's on chromosomes C3, C8, E1 and E8. B. The LOD profiles of the interval mapping analysis for the chlorogenic acid (5-caffeoyl quinic acid) content, showing the observed QTL on chromosome C2. C. The LOD profiles of the interval mapping analysis for tyrosine content, showing the QTL on chromosome C8. The LOD thresholds in all figures have been set at 3.0.

Candidate gene allele identification and mapping

The major candidate gene for enzymatic discoloration is polyphenol oxidase (PPO). The POT32 gene, coding for a tuber specific polyphenol oxidase, was chosen for further analysis. Although the POT33 gene also shows a low expression in tubers, POT32 is likely to be responsible for discoloration as it is the most abundantly expressed member of the PPO gene family in tubers. POT32 gene specific primers were designed on different, but overlapping, sections of the gene and the PCR products were cloned and sequenced. The sequences were subsequently aligned and analyzed. Primer set POT32PS1 was completely POT32 specific, primer set POT32PS2 amplified also sequences of POT33 and POT72, but not of POT32 allele 2. POT32PS2 POT32-specific sequences were selected based on alignment with POT32 sequence (GB U22921) and used to build a contig with the POT32PS1 sequences in order to extend the length of obtained POT32 sequence. Three distinct allelic versions of POT32 were observed in the parental genotypes and designated alleles 1, 2 and 3. Allele 1 is shared between both parents. An alignment of the predicted protein sequence of each of the alleles and of a reference sequence, *U22921*, is shown in Figure 3. Parent C harbours alleles 1 and 2 and parent E has alleles 1 and 3. Allele 1, which is exactly the same as the published sequence of POT32 (GB accession number *U22921*), has 97 % homology with allele 2 on the nucleotide level and 98 % homology with allele 3, alleles 2 and 3 are 98 % identical. In total, in the 990bp sequence, 25 SNP's occur when comparing allele 2 with allele 1, the 25 SNP's give rise to 15 amino acid changes. For allele 3 29 SNP's on 1425 bp sequence are observed which result in 12 amino acid changes. As indicated in the alignment in Figure 3 some of these amino acid changes occur in the relatively conserved Cu-binding domains A and B. One of the changes for allele 2 is in the third histidine of Cu-binding domain A. The histidines in the Cu-domains are very conserved throughout plant species (Marusek et al., 2006). Amino acid changes in this area of the enzyme, will likely have an effect on the activity of the enzyme.

Restriction of the PCR fragments with enzyme *Hph*I, enables the identification of allele 3 because of the absence of an *Hph*I restriction site. The other two alleles do have the *Hph*I restriction site. Allele 1 can be identified using enzyme *Hpy*F10IV, as the allele 1 sequence harbours an extra *Hpy*F10IV restriction site. In this way CAPS markers for alleles 1 and 3 of POT32 were developed and the segregation was analyzed for the complete C x E population and used to map POT32 on the genetic linkage map of C x E. POT32 alleles 1 and 3 map together on chromosome E8. Allele 1 is present in both parents and as such also maps on chromosome C8.

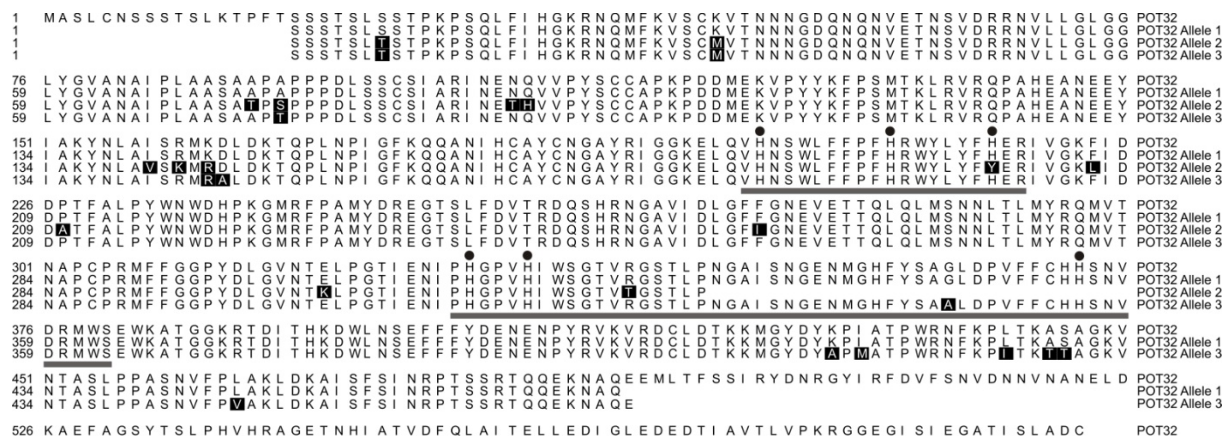

Figure 3: Alignment of POT32 allele protein sequences with POT32 *STU22922* from database. Indicated in black are polymorphisms compared to POT32 *STU22922*. Indicated with black underlining are the two Cu-binding domains A and B (Thygesen et al., 1995), and with the black dots above the alignment the histidines in these Cu-binding domains are indicated.

The effect of the different alleles, on the degree of enzymatic discoloration, can be indicated by grouping all the genotypes of the population according to their allelic composition for POT32 and calculating the median of the enzymatic discoloration scores. An overview of this grouping is given in Figure 4. In the C x E population four different allele compositions can be identified (homozygous for allele 1, allele 1 plus allele 2, allele 1 plus allele 3 and the combination of allele 2 with allele 3). For both time points at which discoloration was measured, the group of clones homozygous for allele 1 showed more severe discoloration. On the other hand, the group of clones carrying alleles 2 and 3 always displayed hardly any enzymatic discoloration. The other two allele combinations show an intermediate phenotype.

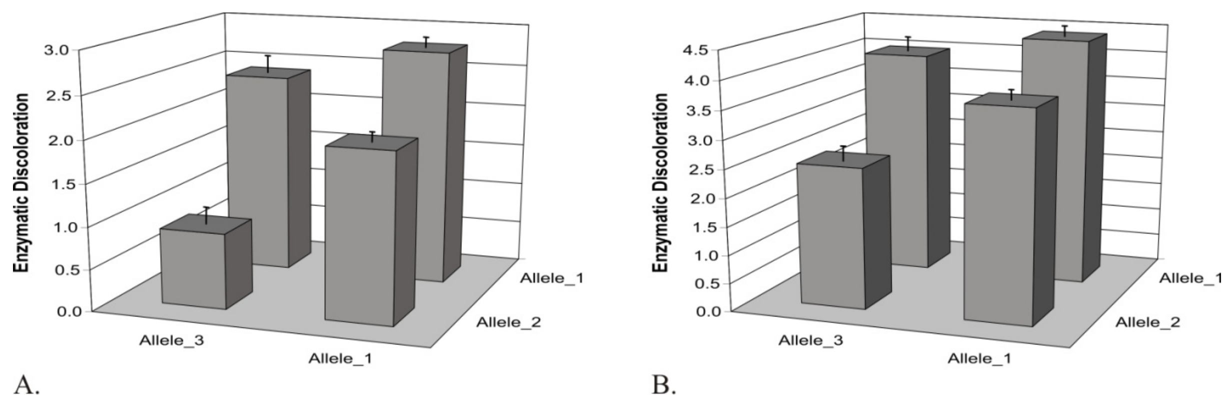

Figure 4: The means of the discoloration scores per allele combination, based on the data of the complete C x E population. The error bars represent the standard error of the means of each allelic combination. Figure A represents the scores at time point 30 minutes. Figure B shows the scores at time point 3 hours after grading of the potato tuber flesh.

PPO expression analysis, using qRT-PCR, was performed on a subset of extreme clones to investigate the possibility of allele dependent differential expression. Due to the high sequence similarity between the individual alleles, no allele specific primers could be designed. Therefore, POT32 gene specific primers were designed in sequence regions for which all three alleles were identical to allow assessment of the sum of transcripts. By using the four different offspring genotypes, the expression levels for allele combinations could be estimated. Figure 5 indicates that the clones homozygous for allele 1 have on average the highest expression levels and the group with a combination of alleles 2 and 3 has on average the lowest levels of expression. This result shows consistency with the previous results for the enzymatic discoloration scores. On the basis of the combined results for the phenotypes and the expression levels, it can be deduced that, on average in this population, POT32 allele 1 seems to have the highest expression levels and its presence relates to a discolouring phenotype. In general, we conclude that variation in discoloration is both due to expression level variation as well as variation in the enzyme activity.

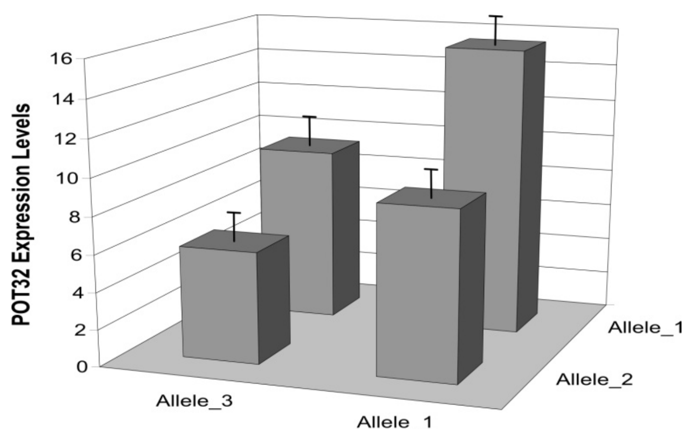

Figure 5: The means of the expression levels for POT32 per allele combination in a selection of extreme phenotype clones for enzymatic discoloration. The expression levels are expressed as fold difference relative to the lowest expressing clone.

Discussion

In this paper we present the genetic architecture underlying the enzymatic discoloration of potato tubers caused by the formation of dark melanin pigments through the oxidation of phenolic compounds by the enzyme PPO. Although it is the first time this trait is analyzed by means of QTL analysis and a candidate gene approach, extensive research has been performed on the trait focussing on both the enzymatic components (Hunt et al., 1993; Newman et al., 1993; Thygesen et al., 1995) and substrate components (Corsini et al., 1992; Marri et al., 2003; Mondy and

Munshi, 1993). The enzymatic component is represented by the enzyme PPO. To explain the substrate components involved in discoloration the levels of two substrates of PPO have been examined, namely tyrosine and chlorogenic acid.
The most obvious candidate gene for enzymatic discoloration is PPO, more specifically the gene POT32. This gene is primarily only expressed in tuber and roots and has the highest expression of the tuber expressed PPO genes (Thygesen et al., 1995). The function of the enzyme in the enzymatic reaction has been well established and is mapped at chromosome 8 of the potato genome (Tanksley et al., 1992). This map position was confirmed by mapping PPO gene POT32 present in the C x E population at chromosome 8, as can be observed from the map of C8 and E8 in Figure 2A.
Three different POT32 alleles in this population could be identified, with 97-98 % homology at the nucleotide level. When translated to the protein sequence, the different allelic forms still share between 95-98% homology. A number of amino acid changes also occur in the active Copper Binding domains, raising the possibility of an alteration in the activity of the enzyme. Figure 4 indicates that the combination of the alleles 2 and 3, or the absence of allele 1, results in a lower degree of enzymatic discoloration. This result is visible at both time points of measuring enzymatic discoloration (30 minutes and 3 hours). Interestingly, the results of the POT32 expression analysis are in agreement with the results shown in Figure 4. Genotypes with the combination of alleles 2 and 3, conferring low enzymatic discoloration, seem to exhibit, on average, lower levels of POT32 expression. This result indicates that besides a difference in enzyme activity of PPO due to sequence polymorphisms, PPO activity may also be regulated at the transcriptional level. Transcriptional regulation may be directly associated to SNP's in the promoter region of POT32, resulting in different levels of expression of the different alleles. However, we can not exclude that the differences in expression levels merely reflect differences in available substrate levels or environmental factors due to a lack of understanding of the mechanisms of transcriptional control of the POT32 gene during the potato tuber life cycle.
The QTL analysis for enzymatic discoloration has indicated the contribution of four QTL's on chromosomes 3 and 8 of the maternal (C) and chromosome 1 and 8 of the paternal (E) map for the trait enzymatic discoloration. Until now we indicated one of the genetic factors underlying the QTL's for enzymatic discoloration, namely POT32 for the QTL on chromosome 8. Two genes underlying other QTL's, ED-1 and ED-3, need to be identified. ED-3 possibly co-localizes with the Y-locus mapped in potato on chromosome 3 (Bonierbale et al., 1988). The Y-locus controls tuber flesh colour and likely candidate genes for this locus are phytoene synthase and/or β-carotene hydroxylase (Thorup et al., 2000). A possible function of these genes, in relation to

enzymatic discoloration, can be deduced from results obtained in apricot (Rigal et al., 2003), where it was observed that enzymatic discoloration could be inhibited by *trans*-β-carotene, either by direct inhibition of PPO or by phenol regeneration through non-enzymatic reactions with quinones. Both genes are involved in the carotenoid biosynthesis pathway. For the QTL ED-1, possible candidates genes can be involved in the production of enzymatic browning inhibitors such as ascorbic acid (Matheis and Belitz, 1977) or thiol compounds such as cysteine (Richard et al., 1991). Besides inhibitors of discoloration, genes involved in the production of phenolic compounds other than tyrosine or chlorogenic acid, may also play a role in the process of enzymatic discoloration such as cinnamic or caffeic acids (Friedman, 1997). OTL analysis of the chlorogenic acid and tyrosine levels resulted in QTL's CA-2 and Tyr-8, respectively. Previous reports indicate a correlation between phenolic content and enzymatic discoloration (Corsini et al., 1992); (Mondy and Munshi, 1993). In our population this correlation could not be observed.
The identification of 3 QTL's within the potato genome influencing the trait of enzymatic discoloration in potato tubers represents a substantial contribution to the understanding of this complex trait. QTL ED-8a can be explained by the PPO gene POT32, the possible relation of ED-3 with the Y-locus could clarify the genetics, underlying this trait, further. The correlation with the substrates tyrosine and chlorogenic acid as described by other authors could not be confirmed in our experiments. Although stated before that selection for PPO activity might not work because of insufficient variation (Brown et al., 1994), selection for the different alleles of POT32 might help in achieving non-discolouring cultivars. Knowledge on the different allelic forms of POT32 and the effect of their presence or absence in the potato genome is a very promising result from a breeding point of view as it allows direct selection on certain alleles in order to reduce the degree of enzymatic discoloration.

Acknowledgements

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Chapter 3

A limited set of starch related genes explain several interrelated traits in potato

Submitted

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Herman J. van Eck, Richard G.F. Visser

Abstract

To understand the molecular basis of potato starch related traits and the underlying starch biosynthesis and degradation, a Quantitative Trait Locus (QTL) analysis in combination with a candidate gene approach was performed. The diploid mapping population C x E, consisting of 249 individuals, was assayed over two consecutive years, for chipping colour, cold induced sweetening, starch content, starch granule size, starch gelling temperature, starch enthalpy, amylose content and degree of starch phosphorylation. QTLs were observed for all traits, except enthalpy on eight out of the twelve potato chromosomes. On chromosomes 2, 3, 5, 8, 9, 10 and 12, QTLs were consistent over two years. On a number of these chromosomes several clusters of co-localizing QTLs were observed, indicating common genetic factors for the different traits. On chromosome 2, Soluble Starch Synthase 2 (SSSII) mapped on the same position as QTLs for starch phosphorylation, starch gelling temperature and amylose content. α-glucan, water dikinase (GWD) co-localizes on chromosome 5 together with QTLs for starch phosphorylation and cold induced sweetening. Furthermore, the genes coding for two phosphorylases (StPho1a and StPho2) coincide with QTLs for starch gelling temperature, chipping colour and starch granule size on chromosome 2 and a QTL for starch phosphorylation on chromosome 9, respectively. The results suggest allelic variation acting on the genetics of the different traits.

Introduction

Starch is the main insoluble carbohydrate in plants and is present in many different plant organs, as transitory starch in green leaves or as storage starch in roots, tubers and seeds (Kossmann and Lloyd, 2000). In potato, starch is mainly accumulated in starch granules of tubers and consists of two different polysaccharides, amylose and amylopectin in a one to four ratio respectively. This ratio has already been found to correlate with a number of different starch characteristics such as retro gradation of starch pastes (Visser et al., 1997b), poor transparency of starch gels and low adhesiveness (Visser et al., 1997a) and high granule melting temperatures (Schwall et al., 2000). Other starch properties including the degree of branching of amylopectin (Kortstee et al., 1998) and the phosphate contents of starch, can also have a profound influence on the characteristics and practical application of starch. Starch properties do not only have an impact on physio-chemical characteristics of starch as gelling agent, they also influence frying quality due to cold induced sweetening of potato tubers during cold storage in a direct manner. During cold induced sweetening, starch is broken down to the sugars glucose and fructose, making the tubers unsuitable for processing (Burton, 1969). The degree of starch phosphorylation has been reported to influence the amount of cold induced sweetening (Lorberth et al., 1998).

In the gene pool of potato a wide natural variation is present with respect to different features of starch, although certain properties can only be achieved through chemical modification. Significant effort has already been put into elucidating the complete biosynthetic pathway of starch granules and understanding the mechanisms underlying the observed variation. Most of the enzymes involved in assembling starch granules are well known. A simplified scheme of starch formation is shown in Figure 1 and proceeds as follows: The substrate for the biosynthesis of starch polymers, ADP-glucose, is produced from glucose-6-P via glucose-1-P by phosphoglucomutase and ADP glucose pyrophosphorylase (AGPase) respectively (Müller-Röber et al., 1992; Stark et al., 1992). ADP glucose is then used by the different starch synthase isoforms to construct the starch polymers. Starch synthases I, II and III (SSSI, SSSII and SSSIII) are mainly present in the soluble phase whereas the granule bound starch synthase I (GBSSI) is bound to the granule and catalyzes the synthesis of amylose (Kuipers et al., 1994a). Other enzymes such as branching enzymes (SBE), debranching enzymes (DBE) and phosphorylating enzymes as α-glucan, water dikinase (GWD) complete the synthesis of starch granules (Hofvander et al., 2004; Ritte et al., 2002). A simplified scheme of starch degradation (Figure 1) proceeds as follows: The first step is the break down of the starch back to the glucoses by AGPases, Starch Phosphorylases (StPho) and amylases, followed by the breakdown of glucose

to sucrose by UDP glucose pyrophosphorylase (UGPase) and sucrose-6-phosphate synthase (SPS) and then the final step from sucrose to the reducing sugars glucose and fructose by invertases (Sowokinos, 2001).

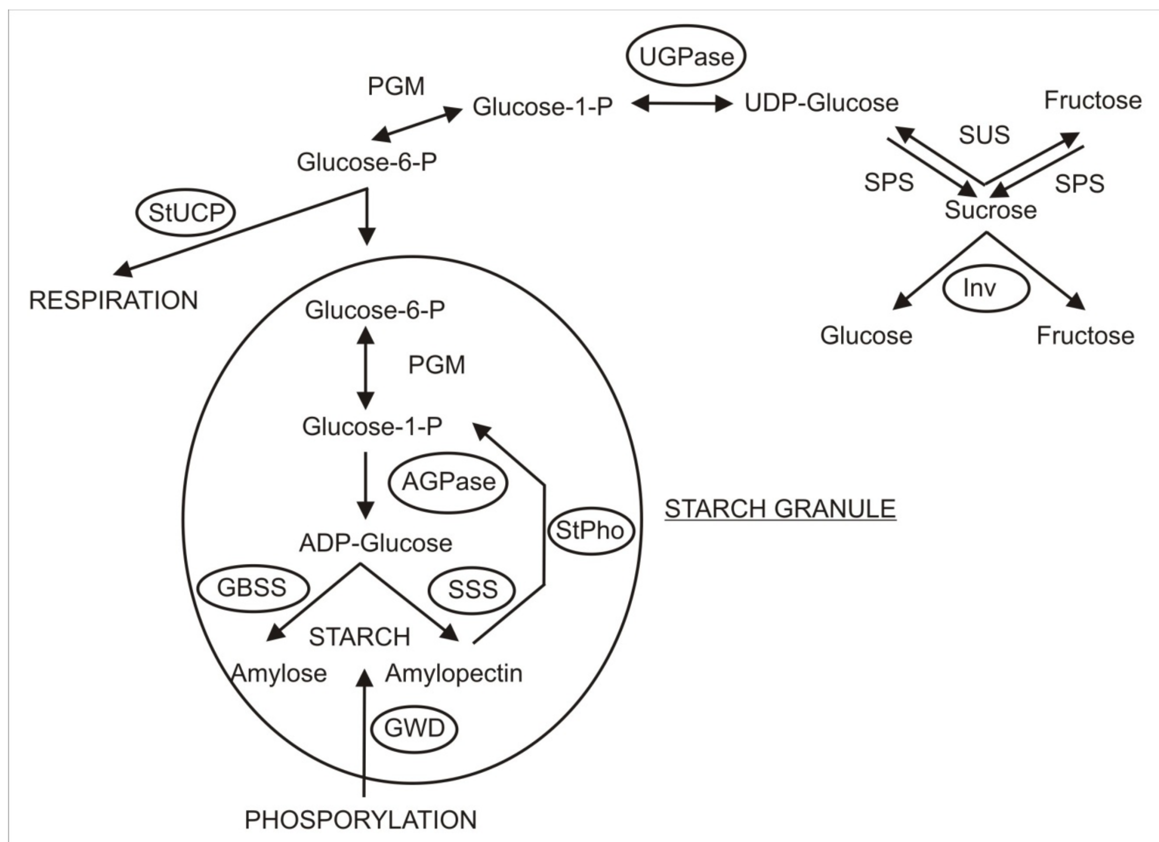

Figure 1: Simplified schematic overview of the synthesis and degradation of starch within and outside the amyloplast showing the following enzymes: UGPase, UDP-glucose pyrophosphorylase; PGM, Phosphoglucomutase; SUS, sucrose synthase; Inv, Invertase; StUCP, starch uncoupling protein; AGPase, ADP-glucose pyrophosphorylase; SSS, Soluble starch synthase; StPho, starch phosphorylase; GWD, α-glucan, water dikinase; GBSS, Granule bound starch synthase starch synthase.

Different sets of genes control the synthesis and the degradation of starch granules and influence potato quality traits. To analyse the influence of these genes on phenotypic variation for starch properties, chipping colour and cold induced sweetening Quantitative Trait Locus (QTL) analysis is an appropriate method. Almost all genes involved have been mapped and cloned, creating the opportunity to strengthen QTL analysis by including candidate gene markers in the analysis. The objective of this work was to identify QTLs for different starch characteristics, for example starch granule size, melting temperature and enthalpy, degree of phosphorylation, amylose content and overall starch content, as well as for chipping colour and the impact of cold induced sweetening on chipping colour. By including markers for the majority of starch related genes into the analysis we were not only able to identify the QTLs, but also indicate which genes

are putatively involved in the different traits. Furthermore, by correlation analysis of the phenotypic data and comparison of the QTL mapping results, correlated characteristics can be identified.

Materials and Methods

Plant material and linkage map construction

The diploid potato mapping population C x E, descending from the backcross between C (US-W5337.3) (Hannemann and Peloquin, 1967) and E (77.2102.37) (Jacobsen, 1980), was used. In this study an expanded version of the linkage map of Van Eck et al. (1995) was used, with an offspring size expanded to 249 full sib descendants and all individuals were genotyped using 16 AFLP markers of primer combinations E32/M47, E32/M48, E32/M49, E32/M50, E32/M51, E32/M54, E32/M59, E32/M61, E35/M47, E35/M48, E35/M50, E38/M59, E39/M60, E45/M59 and E45/M60. The P-locus represents a morphological marker involved in blue anthocyanin pigmentation. Furthermore, the SSR markers STM0003, STM0019, STM0037, STM0051, STM1100, STM2005, STM2028, STM3012, STM3016, STM3023, STM5127, STM5136, STM5148 (Milbourne et al., 1998), and StI001, StI003, StI007, StI022, StI024, StI028, StI029, StI032, StI033, StI046, StI051, StI053 and StI057 (Feingold et al., 2005) were used. SSR markers were amplified in separate PCR reactions in a 20 µl reaction volume, containing 10 ng genomic DNA, 75 mM Tris–HCl pH 9.0, 20 mM $(NH_4)_2SO_4$ 0.1% (w/v) Tween 20, 2.5 mM $MgCl_2$, 100 µM of each dNTP (Fermentas), 4 pmol of each primer and 0.3U Goldstar *Taq* DNA polymerase (Eurogentec). The PCR cycles were 94°C for 3 min, followed by 40 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 45 s and a final extension at 72°C for 10 min. Differently labelled PCR products (6-FAM, HEX and NED) were combined in appropriate amounts to obtain optimal peak patterns for detection. The fluorescently labelled products were separated by capillary electrophoresis using an ABI PRISM 3700 DNA Analyzer (Applied Biosystems). Electropherograms were created automatically using GENESCAN ANALYSIS SOFTWARE v3.7 (Applied Biosystems). Peak mobilities and areas were determined using ABI PRISM GENOTYPER ® 3.6 NT software. CAPS and SCAR markers were essentially derived from Chen et al (2004), Menendez et al (2005), and Li et al (2005, 2008).

The integrated map of the C x E population consisting of 192 dominantly scored AFLP markers, 29 SSR markers and 24 CAPS/SCAR markers calculated as done before (Van Eck et al., 1995). The linkage groups were assigned to the twelve linkage groups representing the twelve chromosomes on the basis of the earlier version of this map.

All clones were grown in field trials on clay soil near Wageningen, the Netherlands during the normal potato-growing season (April – September). Each genotype was planted in four-hill plots, in three replications with randomised plots. The field trials were repeated in four years. The data on potato chipping colour were obtained on the harvests of the years 1998 and 1999, the different starch characteristics were measured on the harvests of the years 2002 and 2003.

Analysis of potato tuber starch characteristics

Directly after harvest of a single plot, 0.5 kg of fresh potato tubers per genotype was peeled, sliced and mixed and grinded. During grinding of the tuber slices, the juice was collected and 0.01% of sodium metabisulphite ($Na_2S_2O_5$) was added. Collected juices were taken to 4°C to settle for at least 3 hours. The juice was separated from the settled starch and demineralised water was added. The starch was mixed with the water and again given the opportunity to settle at 4°C for 3 hours, this washing step was repeated two more times. Finally the starch was poured on a Whatman filter paper on a Buchner funnel and later air dried at room temperature before being powdered using a sieve shaker (Retsch, Germany).

All the starch characteristics were analyzed in three technical repeats of the same starch sample from a single field plot. The starch samples from the harvests of the years 2002 and 2003 essentially represent biological replications, and the final trait value is an average over these three repeats and two years. The starch granule sizes of all samples were measured using a Coulter Counter Multisizer II (Beckman-Coulter, High Wycombe, UK) using a orifice tube. Approximately 10 mg of starch was suspended in about 150 ml of isotonic solution. The measurements of the granule sizes were each time recorded for at least 50,000 particles.

The parameters onset temperature of melting (T_O) and differences in enthalpy (ΔH) were measured automatically by Differential Scanning Calorimetry (DSC), using the Perkin-Elmer pyrus 6 machine (Perkin-Elmer, Vlaardingen, the Netherlands). The characteristics were automatically measured with the accompanying software. The experiment was done as described by Ji et al., 2003.

Measuring the degree of starch phosphorylation (in nmol PO_4/ mg starch) was done using a high throughput 96-well format. 20 mg of starch was put in a glass tube, together with 250 µl 70% $HClO_4$ and heated at 250°C for 25 minutes. Then 50 µl 30% H_2O_2 was added and heated at 250°C for another 5 minutes. After cooling down and the volume was increased to 2 ml by adding H_2O. 100 µl of the starch sample was then put into a 96-well microtiter plate and 200 µl of colour reagent (0.75% $(NH_4)6Mo_7O_{24}.4H_2O$, 3% $FeSO_4.7H_2O$ and 0.75% SDS dissolved in 0.375 M H_2SO_4) was added and mixed for 9 seconds in the microtiter plate reader. After

incubation for 10 minutes at room temperature, the absorbance was measured at 750 nm in the microtiter plate reader against a reference sample. The amount of nmol PO_4 per mg starch could be calculated using the following formula: nmol PO_4 / mg starch = (C microtiter plate x 20)/(Weight x dw%/100)), with "C microtiter plate being the temperature in °C, "weight" the amount of starch in mg and "dw%" the percentage dry weight of the starch.
Amylose content was determined using the protocol by Hovenkamp-Hermelink et al. (1988), by extracting the starch with perchloric acid followed by determination of the absorption at 618 nm and 550 nm after staining with Lugol's solution (I_2-KI).

Starch content (also referred to as underwater weight) was determined for each plot in 1998 and 1999 by calculating a measure for it based on the dry and under water weight. The formula used was (5000/dry weight (grams)) x underwater weight (grams).

Determination of potato chipping colour

The potatoes of each plot of the three replications were harvested and then divided into three equal batches, which makes nine batches per genotype. The three replications of the first batch of potatoes was fried in two weeks after harvest, two other batches were stored in crates in a climate room for three months at 4°C. The three replicates of the second batch of potatoes was fried directly after the three months storage. The three replicates of the third batch was reconditioned at room temperature for three weeks before frying, to see if the potatoes could recover from the cold storage treatment.
For the frying of the potatoes, 3 slices of 3 potato tubers were taken and fried in peanut oil at 180°C until the slices showed no bubbling anymore. Frying colour was visually assessed on a scale from 1 (black) to 9 (cream). The trait values for cold induced sweetening are based on the differences in frying colour observed immediately after harvest and cold storage. The trait values for the reconditioning are based on the differences in frying colour observed immediately after cold storage and after reconditioning.

QTL mapping

The phenotypic data were tested for normality and when not normally distributed, transformed (square root). QTL analysis was performed using the software package MapQTL® version 5.0 (Van Ooijen, 2004). QTL analysis was done using the interval mapping method developed by Lander and Botstein (1989). The results of the interval mapping were compared to the results of the Kruskal Wallis test and no differences between the results of the two analyses were observed.

Detection of a QTL was also done using a LOD threshold calculated with the permutation test option provided in MapQTL® (Van Ooijen, 2004). The two-LOD support interval was taken as a confidence interval for a QTL.

To estimate the cultivar means Y_{ijk} for chipping colour across years the following model was used. $\underline{Y_{ijk}} = \mu + \underline{Genotype_i} + year_j + (replicate\ within\ year)_{k(j)} + \underline{e_{ijk}}$, where (replicate within year) implies the three replicated plots within a field trial from which phenotypes were recorded. The cultivar means for starch phenotypes were obtained on a per year basis using $\underline{Y_{ijk}} = \mu + \underline{genotype_i} + replicate_j + \underline{e_{ij}}$, where replicate within the year of the field trial implies the three technical replicates based on one starch sample. Random terms are underlined. Heritabilities for cultivar means across two years and three plot replicates were calculated according to: $h^2 = \sigma^2_g / (\sigma^2_g + \sigma^2_y /2 + \sigma^2_e/3)$, with σ^2_g the variance component for cultivar main effects, σ^2_y the variance component for the year effect and σ^2_e the error variance. The genotypic main effects from the above mixed model was used to inspect trait correlations.

Results

Phenotypic data analysis

The starch characteristics were measured over two years and averaged per year over three technical repeats. Figure 2 shows the distributions per year for the different starch traits. The traits did not significantly deviate from normality with the exception of the 2002 data for enthalpy. All traits showed a transgressive mode of segregation in both years, as can be seen in Figure 2. In the cases of starch phosphorylation, onset temperature of starch gelling, enthalpy, granule size and percentage amylose, the parental values of C and E are in the middle of the range of the values of the progeny. The level of observed variation of the traits was similar between years; however, we observed a shift in range of the data between years. For instance, the average amount of PO_4 per gram starch was 4.77 mg higher in 2002 than in 2003 but the range of the observations for both years was 25 mg PO_4 (the difference between the highest and lowest of the observed values in each year). A similar shift in range of data was also observed for most of the other starch traits. For the data of 2002 starch phosphorylation, granule size and percentage amylose was on average higher than in 2003, 4,77 mg PO_4, with 1.3 µm and 1.3 % respectively. Onset temperature of starch gelling and enthalpy was higher in 2003, at 0.7°C and 1.0°C respectively. The level of variation in the data is similar in both years for starch phosphorylation being 25mg PO_4, and granule size of 12 µm on average. Very high correlations between years can be found for starch phosphorylation, starch gelling temperature and starch content, with correlation coefficients of 0.88, 0.79 and 0.72 respectively. Enthalpy, average

granule size and percentage of amylose are much less stable between years, with correlation scores of -0.46, 0.47 and 0.45 respectively.

Not only correlations between years within a trait could be calculated but also the correlations between different traits were calculated, as well as the significance of the correlation, as shown by the Pearson correlation coefficient and the corresponding p-values, as shown in Table 2. Several starch characters showed significant correlation coefficients ($p<0.001$). The degree of starch phosphorylation is correlated with starch gelling temperature, average granule size and percentage amylose, with correlation coefficients of 0.42, -0.42 and -0.55. Also granule size and percentage amylose are significantly correlated with a score of 0.34. Based on this data we can conclude that several of the starch characteristics have an effect on one another.

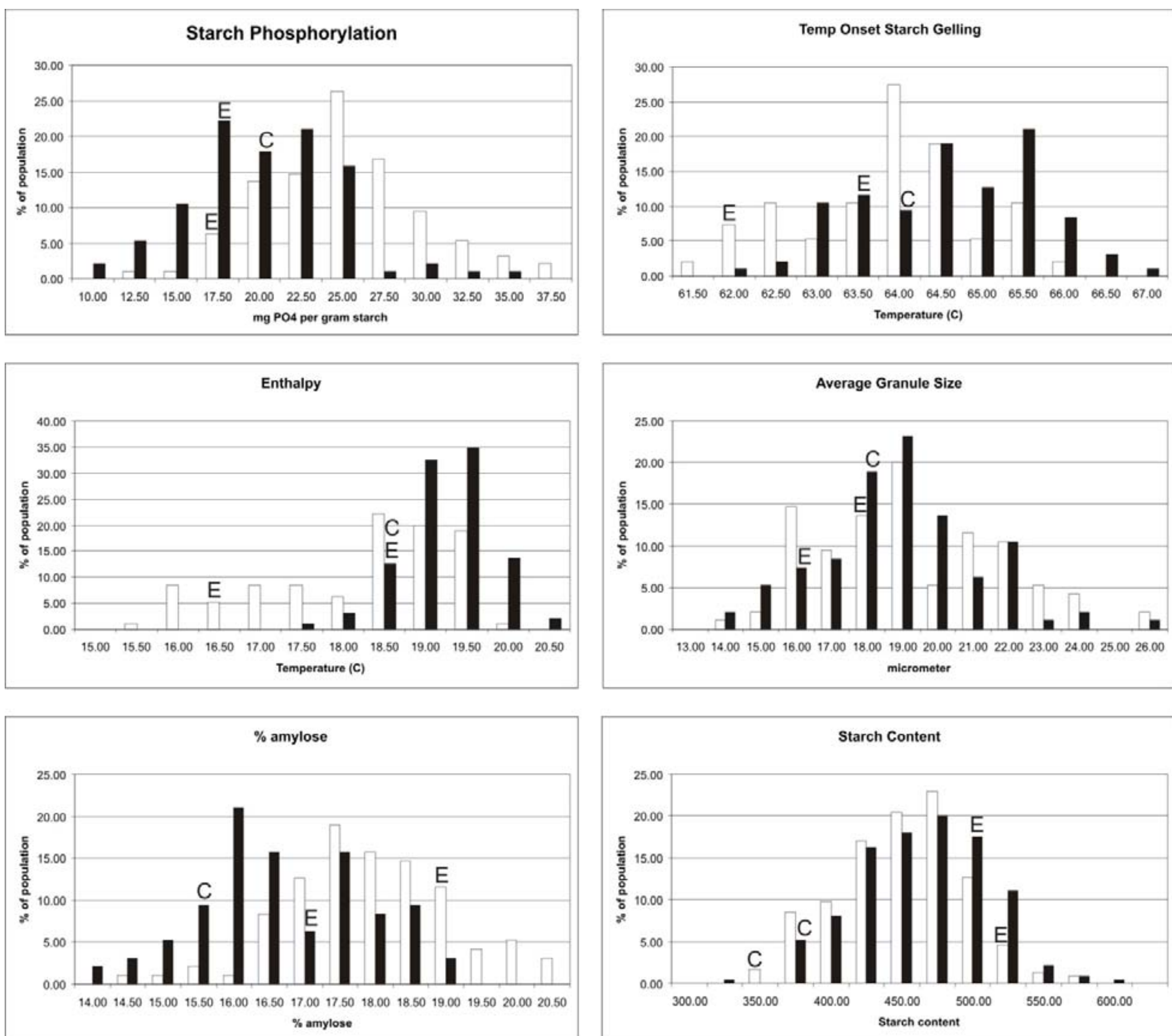

Figure 2: Distribution of the different starch characteristics in the C x E population. The white bars represent the data from 2002 and the black bars the data from 2003, or in the case of starch content 1998 and 1999 respectively. The letters C and E represent the parental values for the individual years. Parental values for C for 2002 are not present due to absence of starch samples.

A

	Gelling Temp.	Enthalpy	Granule Size	Amylose Content	Starch Content
Starch Phosphate	0.42 ****	-0.07 n.s.	-0.42 ****	-0.55 ****	-0.17 n.s.
Gellling Temp.		-0.01 n.s.	-0.23 *	-0.30 ***	-0.02 n.s.
Enthalpy			-0.02 n.s.	-0.04 n.s.	-0.06 n.s.
Granule Size				0.34 ***	-0.07 n.s.
Amylose Content					-0.17 n.s.

B

	Chip-4C	Chip-AR	CS(AH-4C)	CS(4C-AR)	CS(AH-AR)
Chip-AH	0.32 ***	0.42 ****	0.83 ****	-0.04 n.s.	0.88 ****
Chip-4C		0.64 ****	-0.26 *	0.59 ****	0.02 n.s.
Chip-AR			0.06	-0.24 *	-0.06 n.s.
CS(AH-4C)				-0.38 ****	0.89 ****
CS(4C-AR)					-0.17 n.s.

C

	Starch Phosphate	Gelling Temp.	Enthalpy	Granule Size	Amylose Content	Starch Content
Chip-AH	0.07 n.s.	0.13 n.s.	-0.21 *	-0.25 *	-0.22 *	0.35 ***
Chip-4C	-0.29 **	0.19 n.s.	-0.02 n.s.	0.16 n.s.	0.12 n.s.	0.18 n.s.
Chip-AR	-0.12 n.s.	0.10 n.s.	-0.01 n.s.	0.10 n.s.	-0.05 n.s.	0.35 ***
CS(AH-4C)	0.24 *	0.01 n.s.	-0.20 *	-0.36 ***	-0.29 **	0.25 *
CS(4C-AR)	-0.24 *	0.14 n.s.	-0.02 n.s.	0.10 n.s.	0.20 *	-0.14 n.s.
CS(AH-AR)	0.15 n.s.	0.09 n.s.	-0.23 *	-0.34 ***	-0.04 *	0.20 *

Table 2: Analysis of the correlation among and between starch traits and chipping quality traits based on averaged trait values over the years. A. Correlation between starch properties, B. Correlation between chipping traits (Chip-AH = chipping colour after harvest, Chip-4C = chipping colour after storage at 4ºC, Chip-AR = chipping colour after reconditioning at room temperature, CS(AH-4C) = cold induced sweetening based on the difference between chipping colour after harvest and 4ºC storage, CS(AH-AR) = cold induced sweetening based on the difference after harvest and after reconditioning and CS(4C-AR) = difference in chipping colour between 4ºC storage and reconditioning.) C. Correlation between starch and chipping traits. The number of asterix symbols *, **, *, **** denote the statistical significance of the correlation at $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$ and $p \leq 0.0001$, respectively, whereas n.s. indicates the lack of significance.**

Chipping colour and cold induced sweetening were scored for two years. The phenotypic data were similar in both years ($\rho = 0.72$, $p < 0.0001$). A preliminary QTL analysis showed nearly complete coincidence of QTLs (data not shown), which justified averaging across years. The variation within the C x E population with regard to chipping colour is very large. On the scale of 1 to 9, progeny genotypes exhibit scores ranging from 1 to 8. Photographs of the different degrees of discolouration are shown in Figure 3A. The distributions of the chipping colour scores, at the three different time points, as well as those of the values for cold induced sweetening are given in Figure 3B. Just as for the starch characteristics, the chipping colour has an approximately normal distribution and a transgressive segregation at all three time points, see Figure 3B. Broad sense heritability's are estimated to be 0.77, 0.71 and 0.68 for chipping colour directly after harvest, after three months storage at 4ºC and after reconditioning at room temperature respectively. For cold induced sweetening, the heritabilities range from 0.69, for the difference between chipping colour after harvest and after storage, to 0.66 for the difference between the after harvest and after reconditioning time points.

A

B

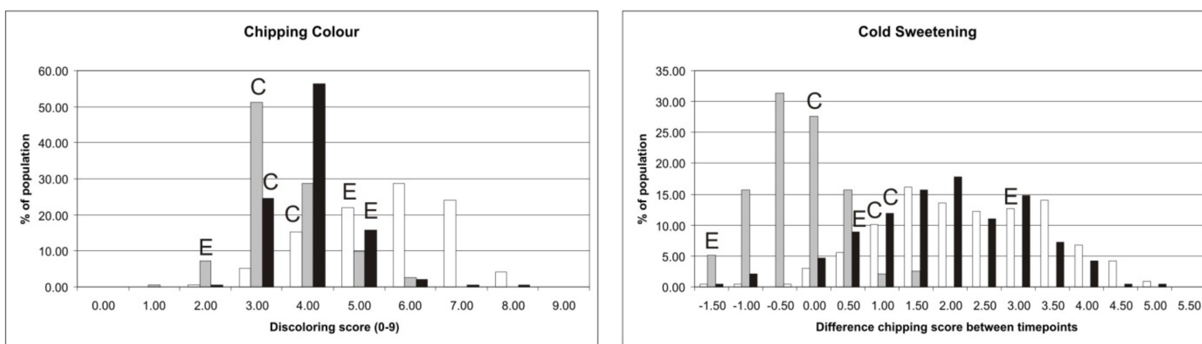

Figure 3: A. Photographs of the different chipping colour scores, ranging from 1 (cream) to 9 (black) B. Distribution of the phenotypic values for chipping colour and cold induced sweetening in the C x E population. For chipping colour (left graph) the white bars represent the colour values directly after harvest (AH), the grey bars represent the colour values after 4ºC storage (4C) and the black bars represent the colour values after reconditioning at room temperature (AR). The white bars for cold induced sweetening (right graph) represent the difference between AH and 4C, the grey bars the difference between AH and AR and the black bars represent the difference between 4C and AR. The letters C and E represent the parental values for the individual years.

In order to establish a relationship between different chipping colours at the different time points and cold induced sweetening, correlation coefficients with associated p-values, were calculated and are presented in Table 2. Significant correlations ($p<0.001$) are found between chipping colour directly after harvest and after reconditioning and between chipping colour after 4ºC and reconditioning. With regard to cold induced sweetening, several significant correlations are observed (Table 2). These correlations exist between chipping colour at different time points as well as between several differences between these time points that we use as an indication for cold induced sweetening.

The results above represent an independent analysis of the starch traits and the traits related to chipping colour. The combination of these two data sets, allows to determine putative relations

between starch and chipping quality traits. The majority of these correlations shown in Table 2 are non-significant, however, with some notable exceptions. A significant correlation ($\rho = 0.36$, $p<0.001$) exists between granule size and cold induced sweetening. Another significant correlation can be observed between chip colour before and after cold storage and starch content ($\rho = 0.35$, $p<0.001$). We conclude that most of the starch related traits, including chipping colour and cold induced sweetening are interconnected.

QTL analysis

All traits were analyzed separately for both years with the exception of the chipping and cold induced sweetening related traits, because no obvious differences were observed between the QTLs for 1998 and 1999. QTLs were observed on eight of the twelve chromosomes using the LOD threshold calculated by permutation test, an overview of the QTL distribution over the map is given in Figure 4. All QTLs were named according to the trait, the year and the chromosome on which they occur. An overview of all the QTLs and their characteristics is given in Table 3.
QTL analysis of starch phosphorylation detected 3 QTLs and were observed in both of the years. The QTLs SP02-2, SP02-5 and SP02-9 explain 21.4%, 15.1% and 19.6% of the phenotypic variance. The corresponding QTLs observed in the other year, explain a larger proportion of the phenotype variance of this trait, (30.3%, 29.0% and 18.1% respectively for SP03-2, SP03-5 and SP03-9). Three QTLs were detected for starch gelling temperature on chromosome 2, 3 and 5. A major QTL indicated as SGT02-2 and SGT03-2 on chromosome 2, explaining 35,8 and 45,5% of the phenotypic variance, was observed in both years. Two minor QTL STG02-5 and SGT03-3 were significant in only one year. For starch granule size, the QTLs on chromosome 8, SGS02-8 and SGS03-8 were consistent in both years, while QTL SGS02-3 is present in 2002 on chromosome 3 and QTL SGS03-9 is present in 2003 on chromosome 9. Amylose content and starch content show the most QTLs of all the starch traits. Five chromosomal regions (2, 3, 5, 7 and 10) are associated with amylose content. The QTLs on chromosomes 2 and 3 were significant in both 2002 and 2003, while the other three QTL were significant in only one year. In total five chromosomes (5, 8, 9, 10 and 12) display significant QTL for starch content, and all QTL are consistent in both years. No significant QTL could be identified for enthalpy in any of the two years.
QTL analysis of the chipping quality and cold induced sweetening resulted in the identification of five chromosomal regions (3, 5, 8, 9 and 10) influencing chipping colour. Chipping colour directly after harvest resulted in two QTLs on chromosomes 9 and 10.

Linkage Group	Trait	QTL	LOD	% expl. var.	Map (cM)	Candidate Gene Locus
2	Starch Phophorylation	SP02-2	3,5	21,4	12,1	*SSSIII*
		SP03-2	5,3	30,3	20,3	*SSSII*
	Starch Gelling Temperature	SGT02-2	12,3	35,8	12,1	*SSSII*
		SGT03-2	13,0	45,5	20,3	*SSSII*
	Amylose Content	Am02-2	6,2	21,9	35,5	*SSSII*
		Am03-2	5,9	17,1	0,0	*SSSII*
3	Chipping color 4C	Ch4C-3	4,1	7,2	16,9	*StPho1a*
	Starch Gelling Temperature	SGT03-3	5,2	20,5	15,3	*StPho1a*
	Average Granule Size	SGS03-3	3,4	22,0	68,9	-
	Amylose Content	Am02-3	5,4	22,2	68,9	-
		Am03-3	4,8	28,5	68,9	-
5	Chipping Color 4C	Ch4C-5	9,5	16,4	46,1	-
	Chipping color after recond.	ChAR-5	7,0	9,6	37,9	-
		CS(AH-4C)-5	6,0	9,8	46,1	*GWD*
		CS(AH-AR)-5	5,0	9,8	59,7	*GWD*
	Starch Phosphorylation	SP02-5	3,1	15,1	47,4	*GWD*
		SP03-5	8,1	29,0	47,4	*GWD*
	Amylose Content	Am02-5	4,4	13,9	37,9	*GWD*
	Starch Gelling Temperature	SGT02-5	6,0	17,5	33,5	-
	Starch Content	SC98-5	11,3	17,3	32,1	-
		SC99-5	3,1	5,0	32,1	-
7	Amylose Content	Am03-7	5,2	13,6	49,4	-
8	Chipping color 4C	Ch4C-8	5,1	8,9	25,1	-
	Cold Sweetening (AH-4C)	CS(AH-4C)-8	4,0	6,8	22,8	-
	Average Granule Size	SGS02-8	7,1	26,0	63,5	-
		SGS03-8	3,6	14,9	57,9	-
	Starch Content	SC98-8	5,6	7,2	20,7	-
		SC99-8	6,0	10,7	33,4	-
9	Chipping Color after harvest	ChAH-9	4,2	8,1	41,6	*StPho2*
	Starch Phosphorylation	SP02-9	5,2	19,6	31,4	-
		SP03-9	4,6	18,1	45,1	*StPho2*
	Average Granule Size	SGS02-9	3,6	20,0	47,0	*StPho2*
	Starch Content	SC98-9	3,1	11,1	5,6	-
		SC99-9	3,2	13,4	0,7	-
10	Chipping Color after harvest	ChAH-10	7,3	12,7	14,9	*StLin8*
	Chipping Color 4C	Ch4C-10	3,6	4,9	45,2	*StLin8*
	Chipping Color after recond.	ChAR-10	3,3	6,8	17,1	-
	Amylose Content	Am02-10	4,0	19,6	3,2	-
	Starch Content	SC98-10	4,3	15,7	17,9	-
		SC99-10	5,2	11,7	18,6	-
12	Starch Content	SC98-12	4,2	9,4	13,6	-
		SC99-12	3,3	8,3	13,6	-

Table 3: Overview of the observed QTLs for starch and chipping traits in the diploid potato mapping population C x E. Starting with the linkage group on which the QTL maps, the trait name, QTL name, QTL significance (LOD score), the percentage explained variance per marker locus, the approximate map position and the co localizing candidate gene locus.

After three months storage at 4ºC and after storage and reconditioning, four QTLs are observed on chromosomes 3, 5, 8 and 10 for chipping after storage and 2 QTLs are found for chipping after reconditioning at chromosomes 5 and 10. The explained variances for chipping colour are considerably lower than most of the starch trait QTLs. For cold induced sweetening three QTLs are detected. Two QTLs on chromosomes 5 and 8, and one QTL at chromosome 5.

Co-localizing QTLs between different years and traits

The QTLs observed in this study are found on eight of the twelve chromosomes of potato. Within these chromosomes the QTLs of different years or traits overlap as shown in Figure 4. All QTLs involved in starch content are consistent between years, which is in agreement with the very high phenotypic correlation between years (ρ=0.72). Equally strong correlations between years were observed for starch phosphorylation and starch gelling temperature, however, the QTLs for the different years of these traits do not always co-localize. For starch phosphorylation this is the case for two of the three QTLs, on chromosomes 5 and 9 and for gelling temperature only one QTL, on chromosome 2, co-localized between years.

On chromosomes 2, 3, 5, 8, 9 and 10 QTLs for multiple traits co-localize, indicating that at these locations there are possible genetic factors that influence multiple starch traits. On chromosome 2, QTLs for degree of phosphorylation, gelling temperature and amylose content overlap. In this case, with the exception of starch phosphorylation, the QTLs for the individual traits are also observed in both years, indicating that starch traits are hardly influenced by environmental variation. At the top of chromosome 3, coinciding QTLs are observed between chipping colour (4º C) and gelling temperature and at the distal end of the chromosome QTLs coincide for granule size and amylose content . On chromosome 5 there is a large group of overlapping QTLs. In this group we find QTLs for starch phosphorylation, gelling temperature and amylose content, but also QTLs for chipping colour at various time points and cold induced sweetening. On this chromosomal region, both starch characteristics, chipping colour and cold induced sweetening traits cluster. Starch content overlaps on the top part of chromosome 8 with cold induced sweetening and chipping colour. On chromosome 9 QTL involved in starch phosphorylation, chipping colour and granule size cluster. The last cluster, on chromosome 10, contains QTLs for starch content, for chipping colour and amylose content at all three time points. From all these co-localizing QTLs for different starch related traits it is clear that the same genes in the starch biosynthesis are involved in various starch and chipping quality phenotypes.

Candidate gene analysis

Fourteen potato genes involved in carbohydrate metabolism have been mapped in the C x E population. After QTL analysis several of the candidate genes were found to co-localize with QTLs for the starch characteristics, chipping colour and cold induced sweetening. An overview of the candidate genes that map together with the different QTLs is given in Table 3, and their co-localizations are shown in Figure 4. Genes SSSII, SSSIII, StPho1a, StPho2, GWD and StLin8 co-localise with two or even three different starch related traits.

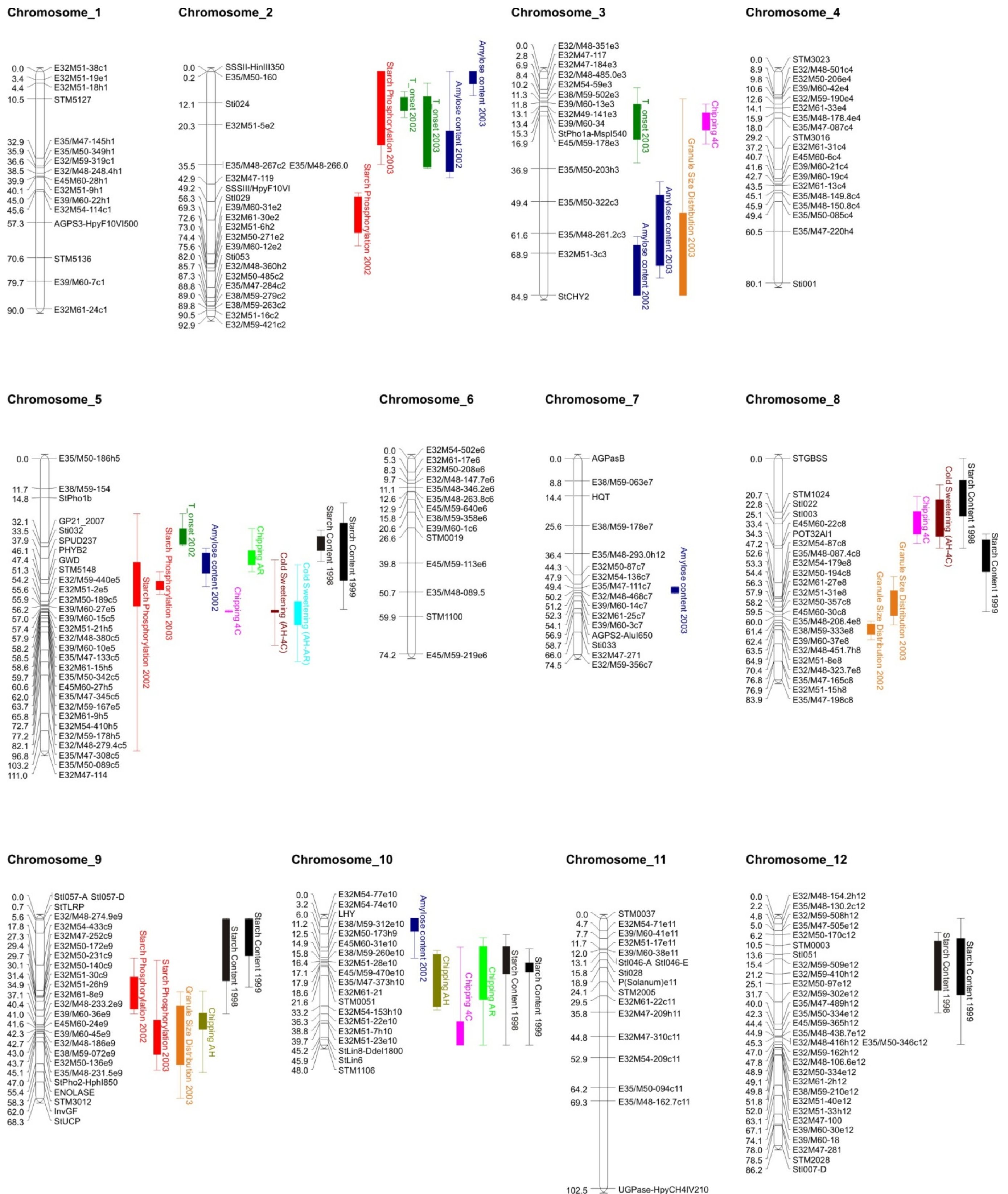

Figure 4: QTL- and integrated linkage map of the C x E population. QTLs are indicated with 2 LOD confidence intervals.

Discussion

Genetic variation for starch properties

This study analysed the genetic variability of starch content in tubers and five important properties of potato starch, namely starch phosphate content, gelling temperature, enthalpy, granule size and amylose content. These starch properties are important for the various applications of starch as gelling agent in several industrial sectors, including food, textile and construction (Ellis et al., 1998). Chemical and physical (rheological) analysis of starches have been reviewed by Singh et al. (2003), but the wide-ranging genetic variability for starch properties caused by the enzymes involved in granule synthesis and degradation, as well as the trait correlations add to our understanding this versatile polymer. The identification of the chromosomal position of a large number of QTLs for different related starch properties, including the co-localisation with loci encoding enzymes involved in starch metabolism, indicate a refined network of interactions involved in starch synthesis. Subsequently, we aimed to understand chipping quality traits as a function of starch degradation into reducing sugars. In total 38 QTLs are observed over different years for all traits. When this number is corrected for co-segregating QTLs, we end up with 13 QTL locations over 8 different chromosomes. Our QTL positions for various starch properties cannot be compared with literature, as these QTLs were not studied before.

The QTL positions for chipping colour in this study correlate well with the QTL positions for chipping colour as identified by Douches and Freyre (1994) and the QTL positions for sugar content and cold sweetening as identified by Menendez et al. (2002). The strong correlation between reducing sugar content and chipping colour was demonstrated by Scheffler (1992). QTLs for chipping colour as observed by Douches and Freyre (1994), Menendez et al (2002), Li et al (2005, 2008), Bradshaw et al (2008) and our study converge to a consensus that major QTL are present in regions on chromosomes 3, 5, 8, 9 and 10, although strict comparisons cannot be made due to the lack of shared markers and map resolution. For the chipping QTLs on chromosomes 3 we confirm the co localisation with starch phosphorylase *StPho1a*, but the invertase locus *PAIN-1* and *SSS-1* have been implicated as well (Li et al 2008). For the QTLs on 5 we propose GWD, while Sut2 proposed by Menendez et al (2002) is also an important candidate. For the QTL on 9 and 10 the apoplastic invertases were proposed (Menendez et al., 2002). Bradshaw et al. (2008) report QTLs for frying colour, after 4°C and 10°C storage. These QTLs are located on chromosomes 1, 6 and 11. These QTLs are not observed in our report nor, with the exception of the QTL on chromosome 1 with AGPasS as candidate gene, in the other report on cold induced sweetening (Menendez et al., 2002).

The putative role of candidate genes on starch properties and chipping quality

On chromosome 5 we mapped GWD, this map position is exactly co-localizing with QTLs for starch phosphorylation and cold induced sweetening. GWD is a clear candidate for cold induced sweetening as it stimulates the breakdown of starch by amylases (Edner et al., 2007) and is previously reported to have an effect on cold induced sweetening via the amount of phosphorus it integrates in the starch granules, in this way affecting the degradability of the granules (Lorberth et al., 1998). The main function of GWD is starch phosphorylation by a dikinase-type reaction (Mikkelsen et al., 2004), making it a strong candidate gene for starch phosphorylation as well. Next to that, GWD is also just in the 2LOD interval of the QTLs for starch content, chipping, amylose content and starch gelling temperature. Based on function, approximate position and previous results (Schafer-Pregl et al., 1998) and the fact that on chromosome 5 a co-localization is reported of the QTL for sucrose and fructose content with Sut2 (Barker et al., 2000), Sut2 is probably the most promising candidate gene for at least the starch content QTLs. Marker development in our research for Sut2, on the basis of known sequence information did not result in a workable marker, suggesting the possible absence or low amount of genetic diversity on the Sut2 locus in this population, therefore we were not able to confirm this hypothesis in our research. However the earlier reports (Schafer-Pregl et al., 1998) reported linked QTLs for starch content on the position of Sut2, suggesting the possibility of two independent QTLs on the upper arm of chromosome 5. For the QTL on chromosomes 9, no co-localizing QTL is observed in the C x E population. In previous reports however, apoplastic invertases were linked as candidate genes to QTLs on this chromosome (Li et al., 2005a; Menendez et al., 2002). On chromosome 9 the invertases were mapped on the upper arm, whereas in our experiments we mapped them on the lower arm of the chromosome. Either the different genetic background or the difference in mapping position could have caused this absence of co-localization. Furthermore, Liu et al. (2011) now firmly have demonstrated the berry specific gene expression or the lack of cold indusibility for the four cell wall invertases on chromosome 9 and 10, which renders these genes unlikely candidates. According to Liu et al (2011) the link between invertase and chipping colour should be explained by the *PAIN-1* (synonymous to *StvacINV1*) locus on chromosome 3 and members of invertase inhibitor gene families at several chromosomal positions. Preliminary QTL analysis on an earlier version of our genetic map, without the majority of the candidate genes, did not show QTLs on previously reported positions, as on chromosome 7. Menendez et al. (2002) reported co-localization of the genes Sps and Sus3 with QTLs for sugar content. Our data clearly shows that the QTLs on

chromosome 7 were absent; it was thus decided not to develop working markers for these genes. For the same reason, no markers were mapped for Sut1 on chromosome 11.

Most of the reproducible and co-segregating QTL effects in the C x E population could be assigned to candidate gene loci. On chromosome 2, QTLs for degree of phosphorylation, gelling temperature and amylose content overlap. For nearly all traits, the QTLs for the different years overlap, indicating a consistent genetic factor regardless of the environment. The LOD profiles of these QTLs co-localize with the map position of SSSII, implying that SSSII is a candidate gene for these traits. SSSII is, together with SSSIII, responsible for the synthesis of amylopectin in the tuber (Edwards et al., 1995). Up to 80% of the starch granule comprises amylopectin making it the most important constituent of starch. As a consequence, changes in the amylopectin production by SSSII, will most likely have an effect on the total amount of starch produced therefore making a relative increase in amylose content possible by a unaltered amylose production. This explains the match of QTLs Am02-2 and Am03-2 with SSSII even though SSSII is not directly involved in the production of amylose itself. On the contrary, GBSSI, located on chromosome 8, is responsible for amylose production (van de Wal et al., 2001). An explanation for the fact that we do not observe a QTL on chromosome 8 could be due to the fact that the alleles for GBSSI present in the population do not show a difference in amylose production. Consequently, no QTL is detected at that position and QTLs at the other positions, such as the QTLs on chromosome 2 at the position of SSSII, likely appear. An earlier article describing antisense plants with reduced activity of SSSII (Edwards et al., 1999) an effect of the reduced SSSII expression on the gelatinisation behaviour of starch was found. This is consistent with the co-localization of the QTLs SGT02-2 and SGT03-2 with SSSII. Effects on the gelatinisation behaviour of starch, caused by differences in numbers and lengths of double helices of amylopectin (Cooke and Gidley, 1992; Moates et al., 1997) are a good indicator for differences in structure possibly caused by allelic differences in SSSII expression. For starch phosphorylation we find QTLs co-localizing in one year with SSSII and in the other year with SSSIII. A clear effect of starch synthases on the degree of phosphorylation is apparent, but there is also an obvious environmental effect shifting the focus between years/environments between both SSSII and SSSIII. Antisense potato lines for SSSII, SSSIII and combined SSSII/SSSIII show clear reductions in phosphorylation levels (Jobling et al., 2002) indicating that both starch synthases have an effect. This effect is possibly caused by the decrease of the amylopectin chain length, reducing the surface to which phosphorus can be attached.

Other genes that appear as candidate genes are two types of starch phosphorylase, more specifically plastidic phosphorylase A (*StPho1a)* and cytosolic phosphorylase (*StPho2*). Both genes are involved in a reversible reaction where a glucose-1-phosphate (G1P) is used as substrate in order to add glucose to the α-glucan chain with the release of phosphorus (Pi). *StPho1a*, in the C x E population, is a candidate gene for onset of starch gelling, chipping and starch granule size. Starch gelling temperature is shown to be affected by the action of Pho1 genes in Pho1 deficient rice plants. It was clearly shown that the onset temperature of starch was significantly lower in the plants with Pho1 deficiency (Satoh et al., 2008). Next to the change in starch gelling temperature in the same rice experiments, a difference in granule size was observed. The Pho1 deficient plants showed smaller starch granules than the wild type plants. This is in line with the results we obtained. Both StPho1a and StPho2 co-localize with QTLs for starch granule size (SGS03-2 and SGS03-9). Furthermore StPho2 maps together with SP03-9, a QTL for starch phosphorylation. We suggest that this may be because of the action of StPho2, by releasing a Pi, it not only reduces chain length but on the larger scale of the whole granule influences the amount of phosphorus that is incorporated in the granule. Lastly, QTL co-segregating with StPho1a is chipping colour, although for a different time point, a QTL for chipping colour is also observed at the location of StPho2. An explanation for this result can be that StPho2 is involved in degradation of starch as it is hypothesised that, although its actions are reversible, phosphorylases favour a role in the degradation of starch (Satoh et al., 2008).

Conclusions

Combined with the already existing knowledge on genes and interactions within the starch biosynthesis and degradation complex, the results of the QTL analysis in this study provide an increasing insight in the genetic components in potato involved in starch traits and chipping colour. The candidate genes found here, are however so conserved that these results can also be of importance in starch related traits in other crop species. The observed marker-trait correlations, like the link between GWD, starch phosphorylation and chipping quality as trade-off suggests that marker assisted selection for specific GWD alleles allow either for the selection for higher starch phosphate levels or improved chipping quality. In general, these results provide a starting point for further investigations and for indirect selection via molecular markers in an effort to improve the existing potato varieties on the traits related to starch.

Acknowledgements

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Chapter 4

Genetic analysis of potato tuber metabolites: In pursuit of improved tuber quality

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Abstract

Metabolites are of utmost importance for the proper functioning and adaptation of plants, therefore they also determine to a large extent the phenotypic traits of importance for human applications. However, much is still unknown about the metabolites themselves and in addition, plant metabolic content is still poorly understood with regard to its genetic basis. Here, we describe the genetic analysis of the metabolic variation present in tubers of the diploid potato population C x E. Metabolic content was analysed using liquid chromatography-time of flight mass spectrometry (LC-QTOF MS). The analysis resulted in a large set of mass peaks, with clear qualitative and quantitative differences. 876 Mass signals were unique for the progeny population and could not be detected in either of the parents. Clustering of the 3024 signals through a multivariate mass-spectra reconstruction strategy revealed 233 reconstructed metabolites (i.e. centrotype) and 425 (14%) single non-clustered mass signals. Quantitative trait locus (QTL) analysis of the centrotypes revealed 803 metabolite QTLs (mQTLs) with a significant LOD score, distributed over all 12 chromosomes. To investigate the contribution of the metabolome to phenotypic traits, the mQTL results were compared with QTL results for tuber flesh colour (raw and after-cooking) and total tuber protein content. For both traits co-localizing mQTLs were observed. The centrotypes linked to flesh colour were identified as being carotenoid derived compounds (glucopyranoside). Carotenoids are amongst the most determining compounds of flesh colour. In relation to protein content, centrotypes were identified as glutamic acid (Glu) and valine (Val). Both are proteinogenic amino acids; compounds that are used as building blocks for proteins and therefore showing a clear relation to the trait total protein content. With this research we offer new insights into the genetics of two potato quality traits as well as proof of principle of a method for unravelling the genetics of quality traits based on their underlying metabolites.

Introduction

The immobile nature of plants compared to other life forms such as animals, forces them to adapt to unfavourable environmental conditions, ranging from biotic threats as pests, diseases and herbivores to abiotic stresses such as drought, heat and cold or nutritional deficiency of soils. Where animals can escape, plants have to deal with the circumstances. The metabolic content of the plant cells is one of the key factors in enabling plants to adapt to all those different environmental circumstances and survive. Next to providing flexibility under different conditions, secondary metabolites also function as basis for plant development and internal physiological housekeeping of plants. This broad functionality of metabolites is facilitated by their immense diversity and number. Currently, 200,000 to 1,000,000 secondary metabolites are estimated to exist in the plant kingdom (De Vos et al., 2007). The wide range in this estimation highlights the lack of firm data on plant metabolites.

In addition to their importance for development and *in vivo* functions of the plant itself, plant metabolism is also of great importance for humans in applications of plants and plant parts for use in biofuel, chemical products, animal feed and human nutrition. Despite the importance of plant metabolites with regard to different human applications, little is known about the metabolite composition. In a model species like *Arabidopsis thaliana* a leaf contains approximately 5000 metabolites of which only a mere 10 percent have been annotated (Bino et al., 2004). Nevertheless, the plant metabolome is receiving ever more attention by plant breeders and researchers in pursuit of enhanced plant performance, as it is clear that quality traits in crop species are highly depending on metabolic composition (Alba et al., 2005).

The huge chemical diversity present in the plant cell, originates from a large amount of different biosynthetic pathways. Until now, the major research emphasis has been on the elucidation of biochemical pathways in the model crop *Arabidopsis thaliana*. Examples include analysis of a specific pathway like the glucosinolate pathway (Kliebenstein et al., 2001) or a more general metabolic profiling approach (Keurentjes et al., 2006). Recently this has been extended to other crop species, like tomato (Gomez-Romero et al., 2010), rice (Heuberger et al., 2010) and cabbage (Yang and Quiros, 2010).

Identification of the actual compounds in plants is merely the first step in fully understanding the functioning of the plant's metabolome. Combining this knowledge with quantitative genetics through performing quantitative trait loci (QTL) analysis, a successful and powerfull approach arises. Understanding the genetics underlying secondary metabolite production can help further unravelling of plant functioning. Several successful studies have shown that this approach is realistic (Calenge et al., 2006; Keurentjes et al., 2006). Although there are some exceptions,

research focuses mostly on specific compound production of well understood pathways. In our research we have taken the broader approach, by investigating a large portion of the metabolic content of potato tubers using LC QTOF-MS. In combination with a QTL analysis of the metabolite data we have gained insight into the genetic factors controlling metabolite production. Through identification of the mass peaks and combining the metabolite QTL (mQTL) results with QTL data from phenotypic traits we not only analyse the genes controlling metabolite production but also the function of these metabolites. See Figure 1 for an overview of the complete methodology used in this chapter. We conclude that this approach offers a useful tool for plant breeders to investigate the genetics of complex traits in dependence on metabolic content.

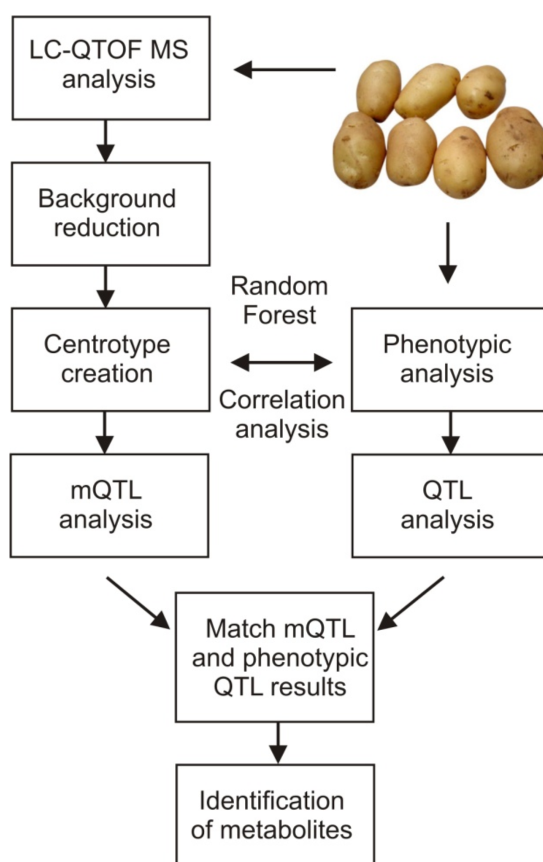


Figure 1: Overview of the followed procedures

Materials and Methods

Plant Material

The diploid potato mapping population C x E, resulting from the backcross between C (US-W5337.3) (Hannemann and Peloquin, 1967) and E (77.2102.37) (Jacobsen, 1980) was used. In the current research a selection of 99 descendants from the C x E cross, out of the 249 full sib descendants (Werij et al, Chapter 3 of this thesis), was made. The 99 full sib descendants were

selected on the ability to produce tubers and were grown in the field (2002, Wageningen, the Netherlands) during the normal potato-growing season (April-September). Of each progeny clone, mature tubers were harvested. Directly after harvest 500 grams of tubers were selected per clone and peeled, sliced and frozen in liquid nitrogen. Then the frozen slides were grinded to a very fine powder in liquid nitrogen and stored before analysis at -80º C.

Phenotypic analysis

For the C x E population a large dataset of phenotypic traits and QTL results exists, which was collected over several years (https://cbsgdbase.wur.nl/static/databases/potato.php). Two potato tuber related traits were used to cross reference the metabolite QTL results and were used for correlation analysis. Both raw tuber flesh colour and after-cooking tuber flesh colour were scored. Both flesh colour traits were visually assessed and graded on an ordinal scale using classes ranging from score 1 to 9 (1 = white to 9 = dark orange) using reference samples. Raw flesh colour was scored directly after peeling. After-cooking colour was examined 5 minutes after the tubers had been in boiling water for about 15 minutes. Colour was scored in three repeats of three tubers from the same sample each. Each sample contained the total number of harvested tubers of one trial field plot, consisting of four plants of the same genotype.
The second trait analyzed was total tuber protein content, using the microBCA assay in two replicates for a selection of 108 clones harvested in 2002 and 2003. The protocol used for the microBCA method can be found at http://abhayjere.com/Documents/BCA.pdf.

LC-QTOF MS analysis

Liquid Chromatography, coupled to high resolution Mass Spectrometry (LC-QTOF MS) was applied to aqueous methanol extracts of the samples. The samples were analyzed for variation in semi-polar metabolite composition using an untargeted accurate mass LC-MS approach, with on-line absorbance spectra measurements using a photodiode array (PDA) detector, essentially as described in (De Vos et al., 2007). In short, 500 mg FW of frozen tuber powder was weighed in glass tubes and extracted with 1.5 ml of 87.5% methanol containing 0.125% formic acid. Samples were sonicated and centrifuged, and then filtered (Captiva 0.45 µm PTFE filter plate, Ansys Technologies) into 96-well plates with 700µl glass inserts (Waters) using a TECAN Genesis Workstation. Extracts (5 µl) were injected using an Alliance 2795 HT instrument (Waters), separated on a Phenomenex Luna C18 (2) column (2.0x 150 mm, 3 mm particle size) using a 45 minutes 5-35% acetonitrile gradient in water (both acidified with 0.1% formic acid) and then detected firstly by a photodiode array detector (Waters 2996) at a wavelength range of

220-600nm and secondly by a Waters-Micromass QTOF Ultima MS with positive electrospray ionization at a mass range of m/z 100-1500. Leucine enkaphalin was used as lock mass for on-line mass calibration.

Metalign software (www.metalign.nl) was used to extract and align all accurate mass signals (with signal to noise ratio ≥ 3) from the raw data files. Signals present in at least 10 samples and with at least one amplitude higher than 100 (about 5 times the noise value) were subsequently selected. Finally, the multivariate mass spectra reconstruction strategy (Tikunov et al., 2005) was used to remove data redundancy by retention time-dependent clustering of signal derived from the same compound, i.e. isotopes, adducts and in-source fragments. From each reconstructed metabolite the signal intensity of the most unique mass was selected for further statistical analyses.

Metabolite identification

Metabolites showing co-localisation of the mQTL results with phenotypic QTLs were (putatively) identified by comparing the MotoDB (http://appliedbioinformatics.wur.nl/moto/) (Moco et al., 2006), Dictionary of Natural Products (www.chemnetbase.com), KNApSAcK (http://kanaya.naist.jp/KNApSAcK) and/or ChemSpider (http://www.chemspider.com). Suggested elemental compositions and annotations were checked for the presence of corresponding in-source fragments in the mass clusters and for absorbance spectra in the original raw data.

Statistical analysis

QTL analysis of centrotype and phenotypic data was done as described in Werij et al, Chapter 3 of this thesis. Random Forest (RF) was used for regression of the phenotypic trait flesh colour (raw) on the metabolomics data set was used. The metabolomics data set was $\log_2$ transformed and then autoscaled (mean=0, sd=1). Tenfold cross validation was used to optimize the number of variables randomly sampled as candidates at each split in the random forest regression trees (the 'mtry' parameter of the R procedure to perform random forest analysis). In each analysis we estimated the variance explained by the RF model (R2) on the so-called out-of-bag OOB samples. These are samples not used for making the regression trees, and therefore this R2 has a different interpretation than the R2 for goodness-of-fit in normal ordinary least square (OLS) regression (Montgomery and Peck, 1992). Variance explained (R2) from RF is a value that is relevant for prediction of independent new samples, whereas the R2 in OLS is a measure for goodness-of-fit of the data at hand. Estimation of variable importance of the transcripts and

metabolites was based on the increase in the mean squared error (MSE) after permutation of the values of the variable. The higher this increase, the more important is the variable in the RF model (Breiman, 2001). Pearson correlation coefficients were used to quantify the strength of association between a combination of a specific metabolite and a phenotypic trait. The threshold for significance used was $\alpha=0.01$.

Results

Metabolic variation

In order to assess the variation in metabolic content within the C x E population we analyzed samples from 99 progeny clones using a LC QTOF MS-based untargeted metabolite profiling analysis. The results showed clear differences in both quantitative and qualitative ways. A total of 14428 mass signals was obtained, this set however contained a large number of low intensity peaks. After performing a background noise reduction, thereby reducing the amount of low intensity peaks, a dataset of 3024 different mass signals remained for the complete population. On average, we observed 2010 mass peaks per progeny clone, with a minimum of 1615 and a maximum of 2419. The distribution of the number of mass peaks per progeny clone is shown in Figure 2A. A clear transgressive segregation pattern was observed with the average parental values of both C and E (1826 and 1803 respectively) close to the mean of the distribution. A total of 876 mass peaks (29%) could not be detected in either of the parents, 1316 mass peaks (43%) were detected in both parents and 832 mass peaks (28%) were present in only one of the parents C or E. The way of calculating background noise reduction prevents us from finding mass peaks that are unique for a specific progeny clone or for one of the parents. Nevertheless, there is a large variation for the occurrence of specific mass peaks in individual clones, an overview of which is given in Figure 2B. We detected three mass peaks that are only present in 10 of the clones and, on the other side of the spectrum, 641 mass peaks that are present in all clones. On average, a given mass peak is detectable in 65% of the population.

Quantitative variation is observed at up to 1500-fold differences, where in some progeny clones the mass peaks are practically absent (no scores above noise level) while the same peaks are abundant in others (data not shown). Reproducibility of the LC-QTOF MS analysis can be assessed on the basis of the results for the technical repeats of the combined samples for parents C and E. Samples of the frozen tuber powder from both parental samples were taken and mixed in equal proportions. Data from these quality control samples indicated that the technical variation in signal intensities was 19.5% on average and that 67% of signals present in the control samples were present in all 8 replicate extracts.

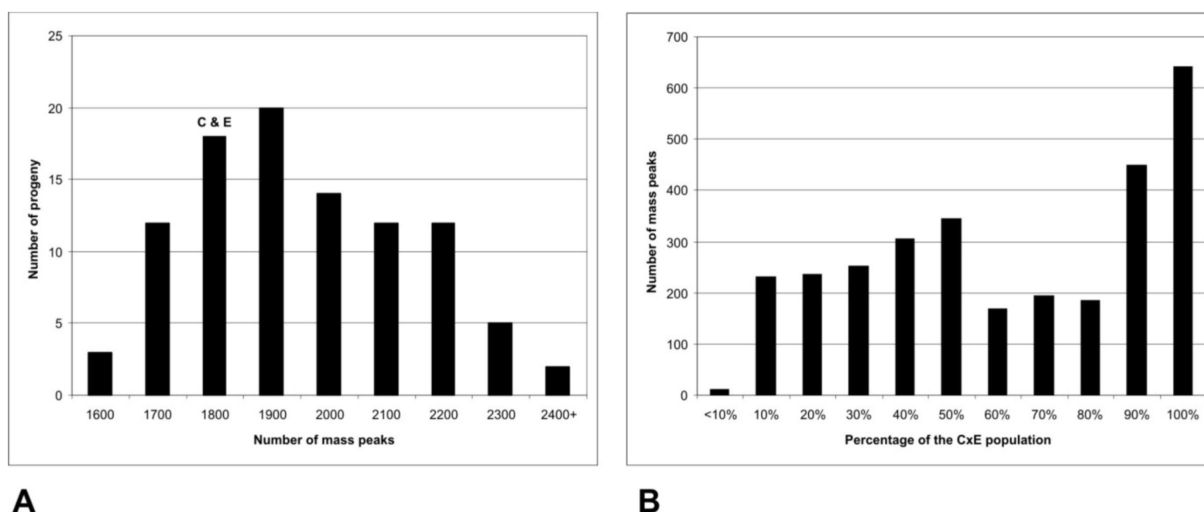


Figure 2: Natural variation in metabolite accumulation in the C x E population A. Frequency distribution of the number of mass peaks detected per progeny clone. B. Frequency distribution of the occurrence of mass peaks in the population.

Representation of reconstructed metabolites

A single metabolite is generally represented by one or more mass signals in the LC MS analysis, differing in chemical structure and abundance. One analysis alternative would be to treat all mass signals as separate data points in further analysis. However we decided in this investigation to group related mass signals into one reconstructed metabolite, representing the original, underlying metabolite. Reason for following this method is to reduce data redundancy (less variables to deal with and fewer highly correlated ones). Different, related mass peaks can often be explained as being fragments, adducts or different isotopes of the same molecule. Therefore, they are often related in chemical composition to the main compound and as such they can be treated as one and the same. This clustering of the 3024 signals revealed 233 reconstructed metabolites (so called centrotypes) and 425 (14%) single non-clustered mass signals. There is a large difference in the number of mass peaks that constitute a centrotype, as can be observed from Figure 3. The numbers range from only one mass peak per centrotype up to 103 masses. A majority (69%) of centrotypes consists of a relatively small number of mass peaks, less than 10 peaks per centrotype.

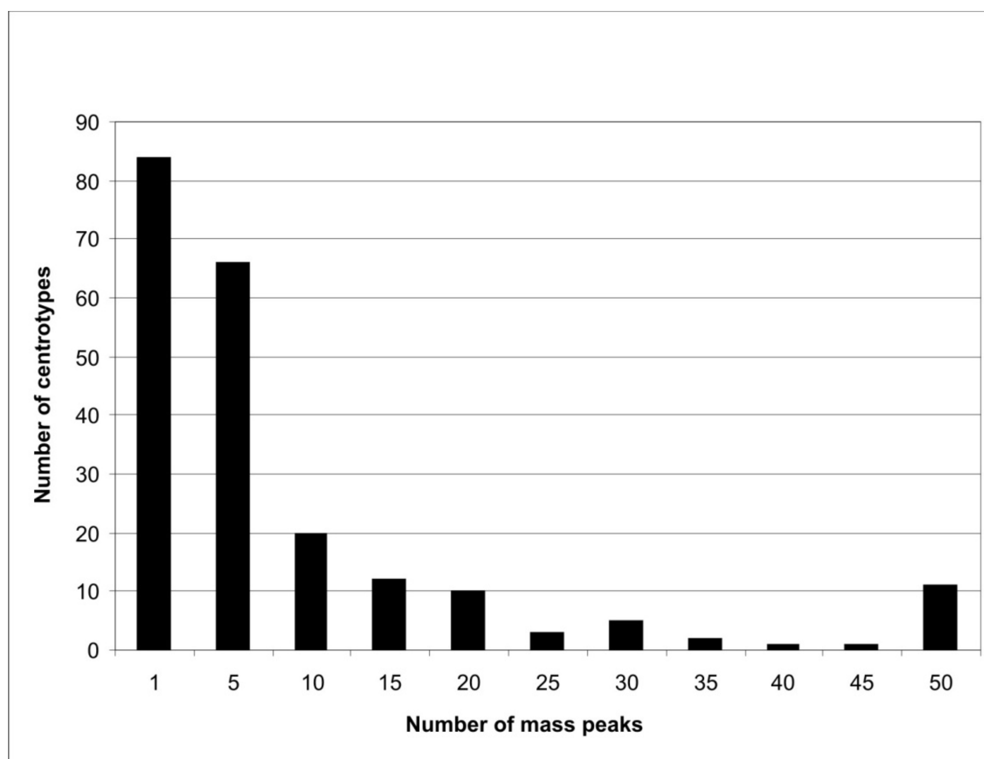


Figure 3: Frequency distribution of the number of mass peaks per centrotype.

Centrotype QTL analysis

In order to uncover loci responsible for the observed variation in metabolic content, we performed a QTL analysis for all 233 centrotypes. This resulted in 803 mQTLs with a significant LOD score (at LOD significance threshold 3.0) distributed over all 12 chromosomes, as shown in Figure 4. For 224 of the centrotypes (96% of all) we observed at least one significant mQTL. The number of mQTLs per centrotype ranges from 0 to 8, and the distribution of the centrotypes resembles a normal distribution (data not shown). On average 3.6 mQTLs per centrotype were obtained.

The distribution of the detected mQTLs shows that these are not evenly distributed over the potato genome (Figure 4). Clear cold and hotspots for the regulation of metabolic content can be observed. Hotspots, with high numbers of co-localizing mQTLs (up to 25 mQTL's per locus), can be observed on chromosomes 1 (90cM), 4 (315cM), 5 (380 and 460cM), 7 (over the whole chromosome), 11 (the distal end of the chromosome) and 12 (950cM). Cold spots, with an absence of mQTLs, can be observed on chromosomes 4 (from 270 to 310cM), 5 (420 to 450cM), 6 (480 to 535cM), 9 (720 to 770cM) and 11 (830 to 860cM).

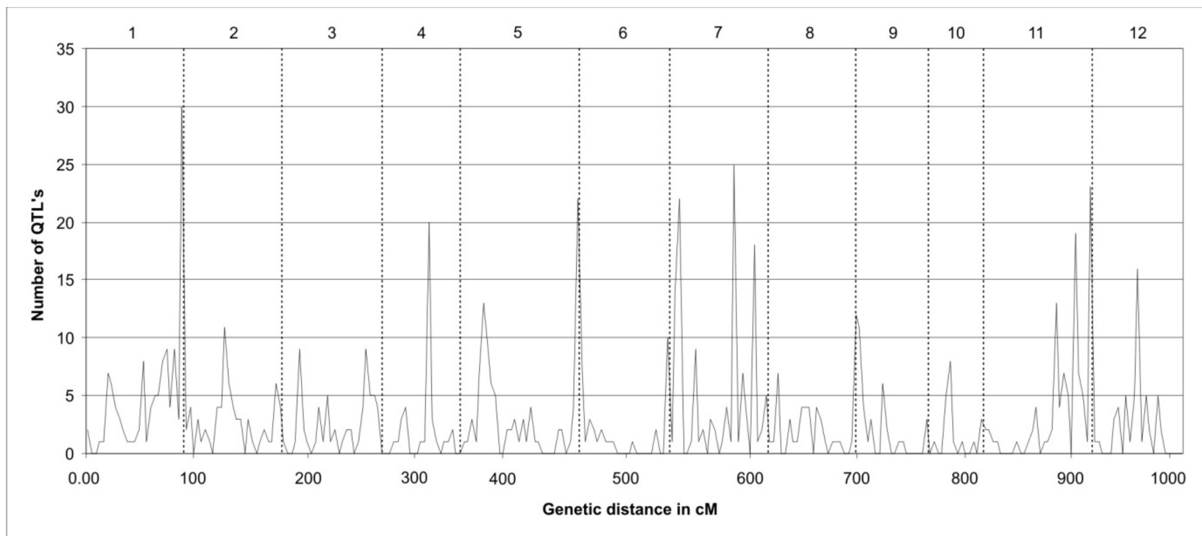


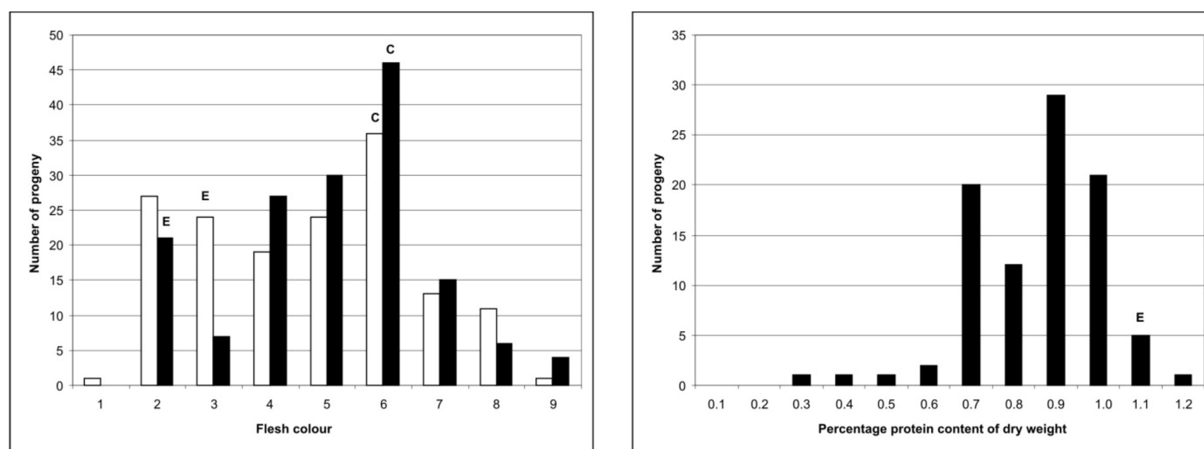
Figure 4: Genome wide QTL distributions of the reconstructed metabolites (centrotypes) on the integrated genetic map of the C x E population. On the top of the graph the chromosome number are indicated. The vertical dotted lines in the graph separate the chromosomes.

Co-localization of phenotypic and metabolite QTLs

To investigate the contribution of metabolic content on potato tuber quality traits, two quality traits were assessed: tuber flesh colour (raw and after cooking) and total tuber protein content. An overview of the data of both traits is given in Figure 5. Flesh colour shows a clear transgressive segregation pattern with parent C having a more extreme phenotype on the left end of the distribution. Progeny clones have more extreme and intermediate phenotypes compared to the parents. Broad sense heritability for flesh colour is estimated to be 0.69. For protein content, the analysis failed for parent C, while scores for other measurements in general had similar scores to that of parent E. The direct comparison of the parental values with those of the progeny to provide evidence for a transgressive segregation was not possible. However, the distribution resembles a normal distribution. Broad sense heritability for protein content is calculated to be 0.56.

QTL analysis for flesh colour resulted in 2 and 4 QTLs, for raw and after-cooking colour respectively. Figure 6 shows an overview of the QTL analysis. The two QTLs for raw flesh colour (explained variances are 8 % and 28% respectively) coincide with those for after-cooking colour (explained variances are 7 % and 21% respectively) and are located around 50cM on chromosome 2 and at the distal end of chromosome 3. The QTLs at the end of chromosome 3, also coincide with a QTL for enzymatic discoloration, see marker E32/M51-3c3 (Werij et al., 2007), which makes a total of three colour related QTLs at the same position. After-cooking flesh colour shows two more QTLs on the distal end of chromosome 1 and at the top of

chromosome 2 with explained variances of 5 % and 15 % respectively. Protein content gives 2 QTLs, on chromosome 1 and chromosome 5 with explained variances of 5 % and 18 %.

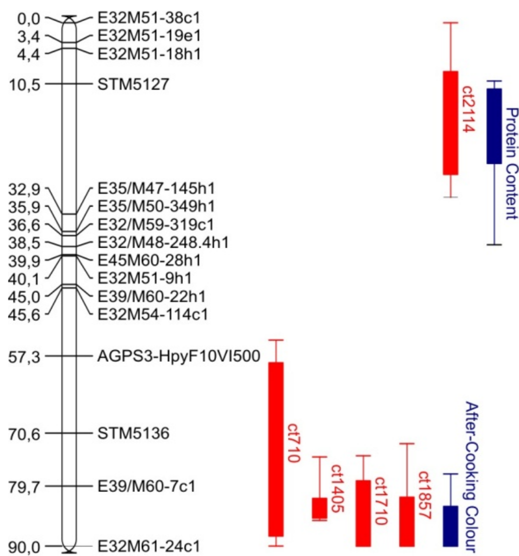


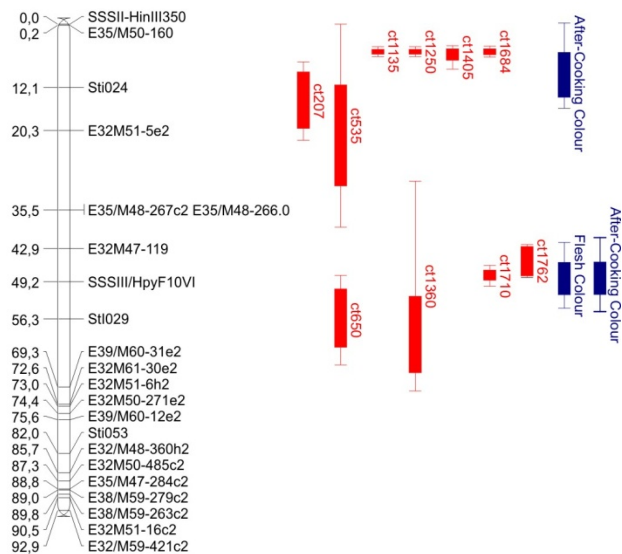
A **B**

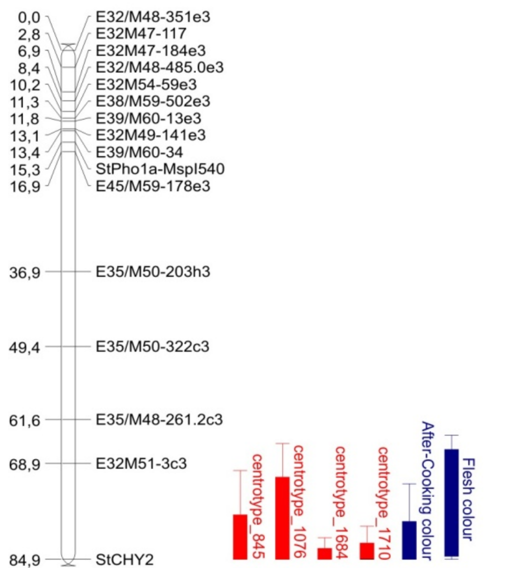
Figure 5: Distribution of the phenotypic trait values in the C x E offspring. Letters C and E above the graphs indicate parental values. A. Flesh colour on a scale of 1 (white) to 9 (dark orange). B. Total tuber protein content on as a percentage from dry weight.

As could be expected with the wide distribution of mQTLs over the potato genome (Figure 4), there are overlapping mQTLs with all QTLs observed for flesh colour and protein content (Figure 6). On chromosome 1, centrotype ct710 shows an overlap with a 2-LOD support interval for a QTL for protein content. On the same chromosome the QTL of after-cooking colour shows co-localization with mQTLs of four different centrotypes. On chromosome 2 there are ten mQTLs co-localizing with the two QTLs for after-cooking colour and four with the QTL for flesh colour. Four mQTLs are overlapping with the QTLs for flesh colour and after-cooking colour on chromosome 5. Finally, on chromosome 5 there are eight mQTLs at the same genetic position as the QTL for protein content. There are four centrotypes that show multiple overlapping QTL results with the phenotypic traits. Ct1405 shows overlapping mQTLs with after-cooking colour on chromosomes 1 and 2. The same holds true for ct1684, which overlaps with the 2-LOD interval of the QTL for after-cooking colour at chromosomes 2 and 3, with also an overlapping QTL for flesh colour at chromosome 3. Ct1710 is overlapping with 3 QTLs for both colour traits at chromosomes 1, 2 and 3. The fourth centrotype is ct535, which is overlapping with a QTL for after-cooking colour at chromosome 2 and a QTL for protein content at chromosome 5. The presence of multiple overlapping QTLs between metabolites and traits might be an indication of the involvement of the metabolite in controlling the phenotypic trait. In some cases, this is supported by a direct, significant correlation between centrotype and trait.

Centrotypes ct1684 and ct1710 show a correlation coefficient of -0.64 and 0.67 respectively with raw tuber flesh colour with p < 0,0005. For ct535 such a correlation exists with protein content (0.34 and p < 0,0005). A total overview of the mQTLs co-localizing with the phenotypic QTLs is given in Table 1, a genetic map of the chromosomes in question is shown in Figure 6.







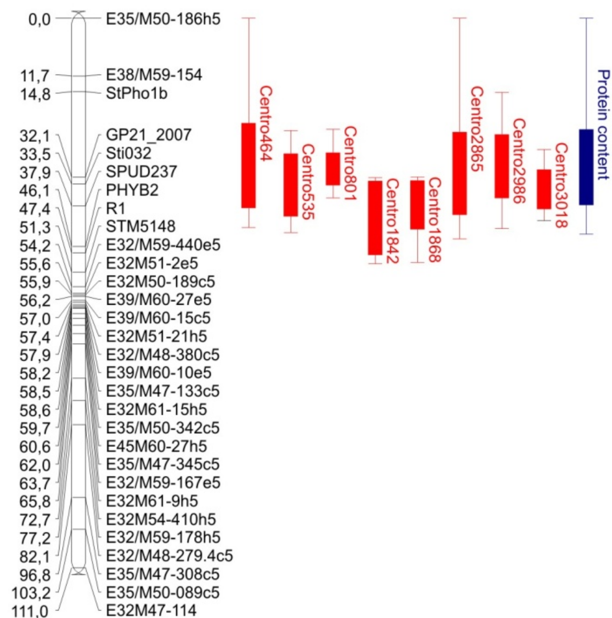


Figure 6: Genetic map of the potato chromosomes 1, 2, 3 and 5 in centiMorgans showing the 2-LOD intervals of the QTL analysis results for the phenotypic traits tuber flesh colour (raw and after-cooking) and total tuber protein content and the QTL results for 22 centrotypes for the chromosomal locations matching those of the phenotypic QTLs.

In order to assess, without the indirect route of comparing QTL results, if there are indications for a more direct relationship between trait and metabolite peak, we checked for association of the phenotypic trait flesh colour with the metabolite data using the random forest model (RF) without using marker information. Where a direct relationship was found, this confirms and supports our findings in the QTL analysis. The variance of flesh colour explained by the RF model for metabolites was 63%. In total seven metabolites were significant at permutation threshold 0.001. The R2 value was also significant at this level. Using only these seven correlated metabolites, the variance explained by the RF model was 77 %. The centrotypes ct207, ct1076, ct1684 and ct1710 were among those seven metabolites and they were also identified via QTL analysis. Those four centrotypes together, explained 70 % of the phenotypic variation. The Pearson correlation coefficients between flesh colour and centrotypes ct207, ct1076, ct1684 and ct1710 were 0,10, 0,70, -0,63 and 0,66 respectively.

Centrotype number	Chromosome	Overlapping trait QTL	Description
ct710	1	After-cooking colour	-
ct1405	1 - 2	After-cooking colour	L-Threonine, Multiply charged ions
ct1710	1 - 2 - 3	After-cooking colour, flesh colour	Multiply charged ions
ct1857	1	After-cooking colour	-
ct2114	1	Protein content	-
ct207	2	After-cooking colour	(Butane, 1-(methylthio)-)
ct535	2 - 3	After-cooking colour, protein content	Citric acid
ct650	2	After-cooking colour, flesh colour	-
ct1135	2	After-cooking colour	-
ct1250	2	After-cooking colour	-
ct1360	2	After-cooking colour, flesh colour	L-Threonine
ct1684	2 - 3	After-cooking colour, flesh colour	Multiply charged ions
ct1762	2	After-cooking colour, flesh colour	Multiply charged ions
ct845	3	After-cooking colour, flesh colour	6,9-Dihydroxy-4,7-megastigmadien-3-one-Glucopyranoside
ct1076	3	After-cooking colour, flesh colour	4,7-Megastigmadiene-3,9-diol, 9-O-?- D-Glucopyranoside
ct464	5	Protein content	-
ct801	5	Protein content	Pantothenic acid
ct1842	5	Protein content	L-Glutamic acid
ct1868	5	Protein content	4-Aminobutyric acid
ct2865	5	Protein content	L-Valine
ct2986	5	Protein content	Tris(trimethylsilyl)hydroxylamine
ct3018	5	Protein content	multiply charged ions

Table 1: Overview of co-localized centrotype QTLs with phenotypic trait QTLs

Centrotype identification

The centrotypes with overlapping QTL results with the phenotypic traits were cross- referenced with the different databases for putative identification. However, not all centrotypes could be

identified. An overview of the description delivered by the databases for each of the centrotypes is given in Table 1. The centrotypes that stand out directly because of a clear link with the respective phenotypic traits are ct845 and ct1076 that were identified as being carotenoid derived compounds (glucopyranoside). Carotenoids being one of the most determining compounds of flesh colour (Kloosterman et al., 2010). Centrotypes ct1842 and ct2865 were identified as glutamic acid (Glu) and valine (Val). Both are proteinogenic amino acids, compounds that are used as building blocks for proteins. Therefore, there appears to be a relation to the trait total protein content.

Discussion

Natural metabolic variation

The approach described here involves the untargeted detection of thousands of metabolites in a mapping population, hereby enabling all the metabolites to be mapped, via mQTLs, on the genetic map. Through the high number of observed mass peaks, 3024 after noise reduction, it is clear that the biochemical variation in potato tubers is extensive. A variation that is both of a quantitative, as well as, a qualitative nature. The quantitative aspect is highlighted by the observation of 641 mass peaks, corresponding to 21% of the total, that are present in the complete population, but clearly differ in abundance. On the other hand, the qualitative behaviour of the mass peaks is obvious when we take into account that on average a given mass peak is present in 65% of the population. Both kinds of behaviour have been observed before in different plant species (Keurentjes et al., 2006; Mitchell-Olds and Pedersen, 1998). Especially the qualitative effects are interesting because of the possible biological consequences involved. Are these centrotypes composed of non-essential compounds, that can either be absent or replaced by others and is this because pathways are interrupted? In contrast to a biological explanation, these centrotypes could also represent technical artefacts and as such have nothing to do with biology. Further investigations into the identity of these compounds are necessary in order to solve these questions.

Apart from the quantitative and qualitative aspects, there is also the balance between effects from the environment and genetic factors on the metabolic content of the population. The large number of observed QTLs, combined with the fact that 96% of the centrotype data results in a QTL, are indications that the metabolic content of tubers is under genetic control. Similar research in *Arabidopsis*, did however result in 31% or 74,8% of the metabolites showing mQTLs (Chan et al., 2010a). The very high percentage (96%) of centrotypes that result in a mQTL in our case, can be caused by the fact that the centrotypes are represented by the signal intensity of a

reconstructed metabolite therefore most probably having a more accurate value than a random mass signal, resulting in such a high percentage. It could be that we selected, by reducing the data set from 3024 mass peaks to 233 centrotypes, the small portion of mass peaks that might actually have a strong genetic basis. We also found a portion of the mass peaks (29%) that were not present in either of the parents. This fraction accounted for 40% of the metabolites in *Arabidopsis* (Keurentjes et al., 2006). This result could indicate that there is a relatively large amount of metabolites in the population that are produced as a result of recombination of the two parental genomes, which is in agreement with earlier findings in *Arabidopsis* (Keurentjes et al., 2006). Although, the way of analyzing the clones in the CxE population (one repeat only) is different from analyzing the parental samples (two samples, six technical repeats each) and therefore the chance of finding more mass peaks around the threshold for the clones is larger.

Even though there is a clear genetic component, it is obvious that also metabolites are environmentally influenced, for instance seasonal variation. Previous reports have shown this as well in other crops like for instance tomato (Tieman et al., 2006). Also in potato, this large environmental effect is reported, where large differences in composition were found between tubers of the same individual cultivar (Beckmann et al., 2007). In our research we compensated for this effect by using a sample taken from several tubers, hereby averaging the levels for each metabolite and reducing the environmental influence.

Apart from genetic and environmental influences, we can not ignore the possibility that some of the observed variation is neither genetic nor environmental, but is of a technical nature associated with the used analytical methods, like preprocessing etc. As stated, there was 19,5% technical variation in signal intensities. This figure leaves enough room for a certain margin of error in the analysis, causing an over estimation of the number of observed mass peaks for any given individual. Would we have included biological repeats into the analysis the number might probably have been lower due to the reduction of false positive scores. The fact that we observe 33 % of the signals present in only a subset of the control samples may lead to the conclusion that we have 33% false positive scores. This will probably be an overestimation as in these cases we are often dealing with low intensity peaks that are in some of the samples just below the detection limits.

Matching QTL results

The QTL analysis of the metabolite data results in a large number of genetic regions of influence on metabolic content. The mQTLs are mapped over all chromosomes and there is a wide distribution of mQTLs over each chromosome. This result is consistent with findings in

Arabidopsis (Lisec et al., 2008) and tomato (Tieman et al., 2006) where a similar distribution of mQTLs over the genome was observed. Associations between one metabolite and several chromosomal regions were discovered, and on the other hand multiple mQTLs at one specific genetic region, so called hotspots. These hotspots contain most probably causal factors for metabolite synthesis or the presence of a regulatory factor at that locus (Chan et al., 2010b). In other plant species, *Arabidopsis* and tomato, similar hotspots were detected after mapping metabolite content (Chan et al., 2010a; Schauer et al., 2008). Opposite, there are also locations without any observed QTL. These "cold spots" can be an indication of inactive chromosomal regions.

Two phenotypic traits were analysed in order to match results with the mQTL results. For total protein content two QTLs were found, at chromosomes 1 and 5. In total nine mQTLs co-localize with the two protein QTLs. Two of which, ct1842 and ct2865, are obvious candidates from a biological function point of view, being glutamic acid and valine. Both amino acids are proteinogenic, building blocks of proteins. Finding these two amino acids can have different explanations. First of all, these amino acids were reported as having some of the highest concentrations in potato proteins (Hughes, 1958). Secondly these two amino acids are crucial in protein development, or at least for the proteins highly influencing total protein content. The QTL analysis for the flesh colour traits, resulted in two QTLs for raw flesh colour on chromosomes 2 and 3 which were identical to the two QTLs on the same location for flesh colour after cooking. Two more additional QTLs for after-cooking colour were observed on chromosomes 1 and 2. The QTL on chromosome 3 is consistent with earlier findings for flesh colour (Brown et al., 2006). Moreover, other reports connect the gene *Chy-2* (beta-carotene hydroxylase 2) with the QTL at this map position (Kloosterman et al., 2010; Wolters et al., 2010). In the genetic map used in our research, the QTL for flesh colour maps directly on top of the marker for this gene, see Figure 6. The mQTLs for ct845 and ct1076 co-localize with the QTL for flesh colour at chromosome 3. After identification of the mass peaks underlying these centrotypes, it is clear that the masses are carotenoid derived compounds (glucopyranosides) and we know that carotenoids are the most determining compounds of flesh colour (Wolters et al., 2010). The identification of these masses closes the circle for this trait, connecting the functional gene to the probable metabolite and the observed phenotype.

The matching results between mQTLs and phenotypic QTLs were confirmed, in the exemplary case of flesh colour, by significant associations, using Random Forest analysis, for four of the centrotypes. To further strengthen the results, these are the four centrotypes, ct1405, ct1710, ct1684 and also ct535 that share multiple QTLs with the phenotypic traits. With the exception of

ct1405, these centrotypes also show a direct correlation with the phenotypic trait. Unfortunately, most of these centrotypes could not be identified thus disabling direct confirmation of the relationship by known function/pathway. Ct535 can be identified as citric acid, although no clear relation can be established between citric acid and the traits under investigation.

Conclusion

The results of this research show that the pathway of investigation that we describe here, from metabolite analysis via centrotype construction to QTL analysis of the centrotype data and identification of the underlying compounds, can result in the unravelling of metabolic pathways responsible for plant phenotypes. For breeders, and in particular for potato breeders, this method gives extra tools for understanding the processes underlying their traits of interest (Fernie and Schauer, 2009). At the moment, the large number of potentially interesting mass peaks are still of unknown composition, but in due time advances in research such as for instance in NMR technology are likely to facilitate the identification of an ever increasing number of compounds. Especially the quality traits under investigation, complex and often multigenic, will benefit of these new techniques by identifying and understanding new metabolic compounds

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Chapter 5

General discussion

Jeroen S. Werij

Introduction

Quality related traits are a loosely defined set of plant characteristics. They include a wide range of traits associated with different purposes and demands from the agricultural chain involved in production, distribution, processing and consumption. In order to achieve an improvement of quality traits, an understanding of the underlying genetics is required. The work in this thesis describes the genetic analysis of a series of potato tuber quality traits ranging from processing qualities such as enzymatic discolouration (ED) (Chapter 2) and cold sweetening (CS) to starch characteristics (Chapter 3), protein content and flesh colour (Chapter 4). The results described here support the understanding of the genetic principles underlying potato quality. These principles and their implications for molecular marker development and ultimately breeding, are subject of discussion.

The genetic basis of quality

In plant breeding the aim is to capture the total sum of genetic variation by means of phenotypic analysis. The phenotypic variation is, however, composed of both the genetic and environmental variation present within and around the plant material of interest. The ratio between genetic and environmental influence on a trait determines the applicability for improvement by breeding. A sufficiently large genetic component is therefore of very important. The heritability is indicative for the genetic component of a trait. In Chapter 2, enzymatic discoloration shows a high heritability of 0,84 and 0,82 at two time points. An even higher score was observed for tyrosine content of 0,88. In Chapter 3, the heritabilities chipping colour and cold sweetening over the different treatments were on average 0,72 and 0,68 respectively. These relatively high heritabilities are confirmed by another report on cold sweetening (Menendez et al., 2002). For protein content and tuber flesh colour heritability scores are also high. Results for the individual carotenoids underlying flesh colour are reported to be similarly high with scores from 0,51 up to 0,93 (Haynes et al., 2010). Clearly, these are all examples showing that the genetic basis of many quality traits is strong. Furthermore, we observed a high reproducibility of phenotypic performance of the material used in our experiments. Observations were performed over two consecutive years for most of the traits under investigation and in the majority of the cases a strong correlation was observed between the year-to-year repeats.

Another aspect of genetics of quality traits is the quantitative nature of many of the traits. For instance, of all the 18 individual quality traits investigated in this research, only 3 traits do not show multiple QTLs or no QTLs at all. The number of observed QTLs ranges from 2 to 5 per trait. The number of multiple genetic factors influencing the trait of interest indicates a complex

inheritance pattern. For quality traits this may be common but it is in strong contrast with many of the monogenic characters such as disease resistances of interest to breeders in potato. For every resistance gene in the plant there is a corresponding avirulence gene in the pathogen, limiting the genetics to one gene at the time (Flor, 1971). Due to the multitude of genes involved, in quality traits, the resulting phenotype is often based on genes acting together in complex metabolic networks, such as cold sweetening where both the starch biosynthetic as well as the starch degradation pathway play a role in the observed variation for cold sweetening (Chapter 3). Within these biosynthetic pathways there is a certain level of redundancy where individual steps can be influenced by several enzymes, thereby only showing a clear effect when either all of the genes or a single key-gene involved are targeted. In Chapter 3, examples of these key genes are GWD and SSSII that act as crucial factors for several different traits, illustrated by the co-localizing QTLs on chromosomes 2 and 5. Furthermore, for GWD this is also proven by a GM approach (Lorberth et al., 1998). In addition, invertases were also shown to be key genes in the process of cold sweetening (Bhaskar et al., 2010; Greiner et al., 1999).
Another complicating factor in the genetics of quality traits is the fact that it often comes down to the presence of a specific allele in order to get the desired phenotype. A clear example of this principle is flesh colour where out of eleven different alleles of the *Chy2* gene, only one turned out to be responsible for the difference between yellow or orange flesh colour (Wolters et al., 2010). The same principle holds true for the results described in Chapter 2, where the homozygotic condition of one particular allele of POT32 caused the highest degree of discoloration. This dependence on a specific allele to achieve the desired phenotype is a key factor in breeding for quality traits. In conclusion we can state that there is a solid genetic base for quality traits, which is often quantitative in nature and allele specificity is the key to top quality.

Marker assisted breeding for quality

The ultimate goal of this research is to develop or facilitate the development of molecular markers aiding in the selection of quality traits in potato. The starting point for the creation of molecular markers is the region that influences the trait of interest. For the traits under investigation in this thesis multiple QTLs were identified, as previously noted. These QTLs can only become useful in marker- assisted selection after translation into diagnostic markers with validated predictive power for each trait. There are different aspects that will determine if a marker/QTL can be used for selection purposes.

A first step in unravelling the predictive power is the explained variance resulting from the QTL analysis for each QTL or underlying marker. For the traits analyzed in this thesis values for explained variances were observed ranging from 7,2 % for one of the QTLs for chipping colour at 4°C to 45,5% for one of the two starch gelling temperature (SGT) QTLs. Several of these QTLs had a considerable value making it suitable as a predictive tool for trait selection. A marker based on the SGT QTL will have a sufficient predictive power. However, a drawback of the quantitative nature of quality traits with respect to marker development is that the total explained variance has to be divided over several QTLs. Especially in the cases were there are up to 5 QTLs observed and per QTL the explained variance is relatively low, multiple markers will be needed to select for the trait and the predictive value of each individual marker is relatively small.

The molecular markers used in this research are of three different types, AFLP, SSR and CAPS. The use of these markers underlying the QTLs as predictors for trait selection depends heavily on the type of marker. The majority of the markers in this thesis consist of AFLP markers which are randomly distributed and the underlying sequence is unknown. SSR's have a known sequence and because of their previously known (published) location they are non-randomly distributed (Feingold et al., 2005; Ghislain et al., 2004). Finally, CAPS markers have a known sequence and are mostly based on the genome sequence of known candidate genes, sometimes including the polymorphic SNP (for instance POT32 in Chapter 2) and are therefore very informative. The different information content of the various marker types makes them more or less suitable as selection marker. The information content of the anonymous AFLP markers in particular, can be drastically improved by identifying the specific SNP that caused the marker-trait association. A clear procedure for the conversion of AFLP to SNP is published (Brugmans et al., 2003). Creating specific SNP based markers greatly enhances the predictive power for the trait and of the markers under the QTL observed in this research. The availability of ever more genome sequences will boost the development of SNP markers. The development of SNP markers, based directly on the observed polymorphisms in the genome sequences will result in large amounts of potential useful molecular markers for breeding. This principle has already proven itself in other crops like soybean (Hyten et al., 2010). Candidate gene markers offered in the different chapters have, in several cases, proven their link with the trait of interest. Markers for POT32 (Chapter 2), SSSII, SSSIII, StPho1a, StPho2, GWD and StLin8 (Chapter 3) show links with traits based on genetic position and known function.

A last factor of importance with regard to the use of the results in this thesis for marker-assisted breeding is the presence and spread of the observed genetics in the available potato breeding

material of the different companies. In other words, is the genetics observed here specific for this population or is it applicable in other, non-related material as well? A trait for which this phenomenon is reported is tuber shape (Chapter 1). For tuber shape a single locus on chromosome 10 was mapped with a dominant allele Ro conferring round tuber shape (Van Eck et al., 1994a). Other reports using populations with different genetic backgrounds mention QTLs on chromosomes 2, 5 and 11 (Bradshaw et al., 2008), 2 and 11 (Śliwka et al., 2008) and 7, 12 and an unassigned linkage group (Sørensen, 2006). So clearly, there are more factors controlling the trait, probably depending on the genetic background that is used, as different combinations of *Solanum* species were used to create the respective populations. To what extend this principle is valid for the C x E population in which our research was done has to be determined. This can only be done by thorough analysis of commercial potato material or breeding material with the markers proposed in this thesis. If we compare one of the traits from Chapter 3, chipping colour after 4°C storage, with published results we find remarkable similarities, although direct comparison is difficult because of absence of common markers. QTLs Chp4C-3, -5, -8 and -10 co-localize at the approximate positions of QTLs for baking and frying colour found by association mapping in a set of 430 tetraploid varieties (D'Hoop, 2009). Other QTL results are confirmed by results in other genetic material published in literature. The link of candidate gene POT32 in Chapter 2, was already implied in earlier research (Thygesen et al., 1995) and the results for starch related traits in Chapter 3 partly corroborates previous reports (Menendez et al., 2002). Based on the explained variances and the confirmation of results in a broader genetic set of material, combined with the potential of the candidate genes and starting point for marker development offered by the AFLP markers, the results in this thesis offer a good start for marker assisted selection.

~Omics applications in search for quality genetics

The classical way of investigating genetic principles by creating a population and performing QTL analysis, even supported by the application of candidate gene analysis, gives clear results as proven by the different chapters in this thesis. Traditionally, positional cloning through fine mapping can reduce the amount of candidate genes for further testing. Crucial in these approaches is a certain amount of prior knowledge with regard to pathways or regulatory steps. Even though QTLs can be identified we are, without the prior knowledge, often not able to identify the genes underlying the QTLs. However, in the past decade several new scientific tools have arisen which can aid in the discovery of new genes and gene functions.

The three principles that are most interesting from a marker development point of view are genomics, transcriptomics and metabolomics. From the field of genomics it is the genome sequences that will improve marker development drastically. Recently the genome sequence of potato came online (http://potatogenomics.plantbiology.msu.edu/), opening up new possibilities for marker development (PGSC, 2011). Linking up genetic maps, via physical maps to the genome sequence or via online sequence comparison of the primer sequences for your SNP markers co-localizing with your QTLs can give you the region controlling the trait of interest. Identification of the different genes in the area between two flanking markers of your QTL for instance, can result in new possible candidate genes. With more and more genomes sequenced and following in that trail, more and more gene annotations available, it will be possible to find putative functions for your newly found genes based on sequence similarity with known genes from other species. To add extra information in the quest for finding candidate genes, metabolomics can offer aid from another angle. Several options exist in exploiting the metabolomics functionality. As metabolite levels in plants are also of a quantitative nature, QTL analysis can be applied directly on the data resulting in so called m-QTLs (Keurentjes et al., 2006). This can be done for specific compounds, like vitamins or antioxidants but also in a more general way on the complete metabolic content of the plant. The mQTL data can be combined with the data from trait QTL analysis in order to find co-localizing QTLs indicating identical genetics (Chapter 4). If these broad metabolomics approaches are combined with the set up of a bulk segregant analysis (BSA), bulks of plants with similar phenotypes can be used in order to pick up the metabolites that can discriminate the different groups. In a similar fashion transcriptomics can be used, only in this case to identify the genes that are differentially expressed between the different batches of phenotypes (Kloosterman et al., 2010). In Chapter 2, we used a similar approach to look for difference in expression between bulks of different genotypes. The use of micro-array technology can further specify which potential candidate genes are of interest in a specific population (Kloosterman et al., 2008). Just like with the metabolite data, transcriptomics data can be regarded as a quantitative trait and after genetic mapping result in so called eQTLs. Different approaches of transcriptome profiling can be used. Next to the above mentioned BSA approach, profiling of wild-type versus mutant lines can provide information on functions of specific genes, different developmental stages can be correlated with the activation or inactivation of genes and candidate gene lists can be prioritized based on their expression patterns (Klee, 2010). One of the limiting factors for gene isolation, however, has been the lack of information with regard to plant secondary metabolism. Direct correlation of metabolic data with genotypic data can lead to a QTL analysis using the genetic

map to pinpoint the genetic locations influencing metabolic function (Keurentjes et al., 2006).The results can be used to identify co-regulatory networks and pathways influencing the trait of interest based on the co-expression connected to the trait.

When the different disciplines (genomics, transcriptomics, proteomics and metabolomics) are merged they can greatly enhance the power for new gene and network discovery. An overview of how a marker development pipeline could look like is shown in Figure 1. The integrated approach will give a complete picture of which genes are expressed at what time point and as a consequence resulting in which metabolites. This complete understanding will provide a powerful tool for genetic marker development. In potato this principle is proven, which has lead to new understandings about the anthocyanin production in potato tubers (Stushnoff et al., 2010) and improved beta-carotene production in potatoes (Diretto et al., 2010).

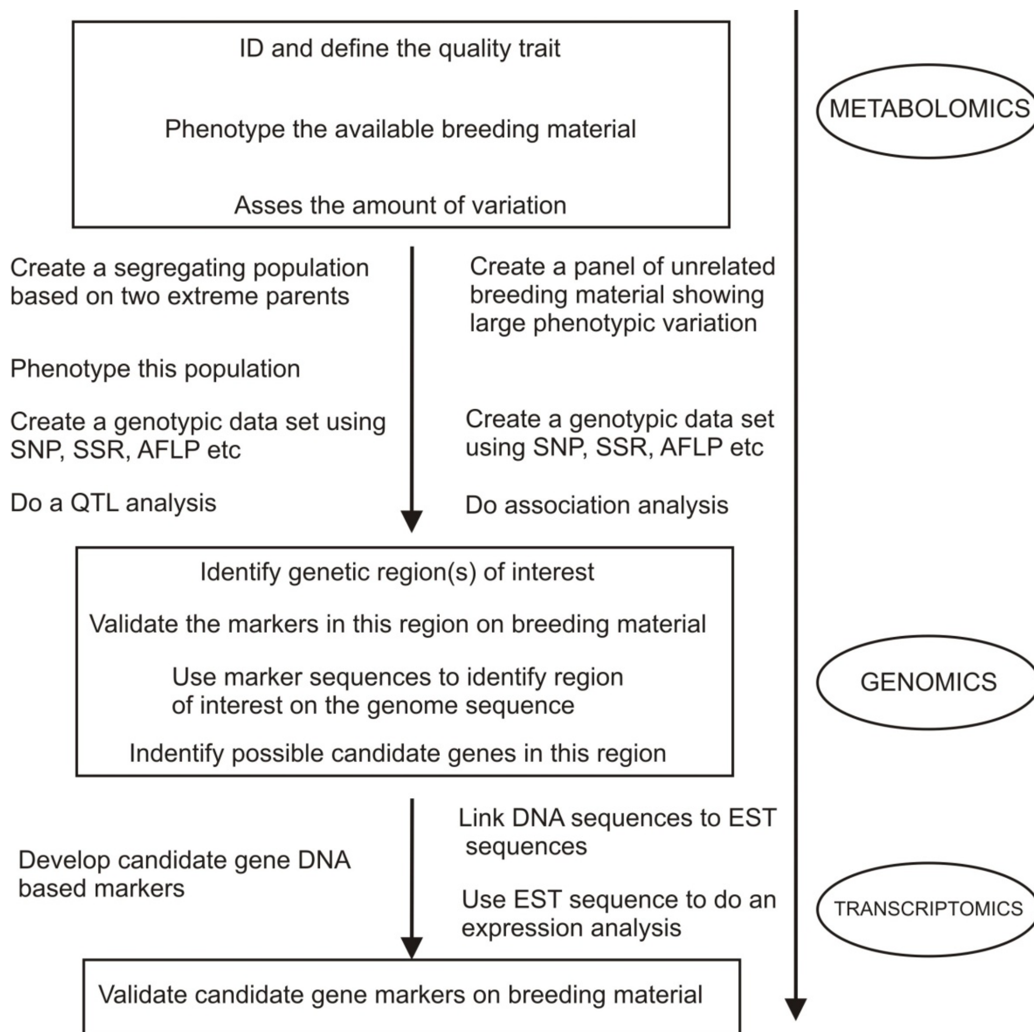

Figure 1: A schematic overview of the potential setup of a marker development pipe line.

Improving quality through genetic modification

Described above is one of the two broad approaches to manipulate the genetic content of breeding material. The other way is through a transgenic approach. For instance final proof of gene function can be obtained by genetic modification of plants with the gene of interest, where plants without the desired phenotype exhibit the phenotype after transformation or vice versa. The function of several potato genes has been confirmed in this manner, amongst these are *GBSS* (Visser et al., 1991), *SBE A* and *B* (Schwall et al., 2000) and *Zep* (Römer et al., 2002). In order to gain the desired phenotype another option is to integrate a gene with known metabolic function from another organism. In potato this strategy was used to increase the levels of vitamins A and E, which do not occur at high levels in wild type potato (Crowell et al., 2008; Diretto et al., 2010). Another example is the modification of potato with an *Escherichia coli* maltose acetyltransferase (MAT) gene to get improved starch acetylation which is of importance for chemical derivatization of starch (Nazarian Firouzabadi et al., 2007).

One of the greatest advantages of genetic modification in a practical sense, is the scientific proof of gene function. In particular, when applied to a specific crop where that gene may not have existed before. This technology can create a completely new phenotype/genotype combination for the crop in question. Although the benefits of the GM approach are clear the negative public perception of the technology prevents major breakthroughs in breeding using this approach. GM varieties are still not widely accepted and combined with the strict legislation (in the European Union) makes that there are only few successful GM crops, and hardly any with transgenic improvements of quality. One of these improvements however is in potato, where plants with reduced amylose content are now at the brink of being commercialised in Europe (http://gmoinfo.jrc.ec.europa.eu/gmp_browse.aspx). Changing the public opinion towards GM crops will not go fast, so large numbers of GM crop introductions can probably not be expected any time soon. However, in the end the advantages of GM crops may be too great to ignore. Environmental issues, like proper processing of industrial waste, could be solved by GM approaches as demonstrated by the above example of the MAT gene from E. coli (Nazarian Firouzabadi et al., 2007). Another argument that might change the public perception towards GM, is the growing pressure on food production by a growing world population and the decreasing agricultural area. For these problems genetic modification might turn out to be the solution.

Prospects of breeding for quality

Although already started years ago, breeding for quality will no doubt, take extra momentum in the coming years. Over time we have learned more and more of the underlying pathways and networks of the wide variety of quality traits. The strong genetic basis for most traits, the growing grip on the quantitative genetics and the broad application of these principles provide an excellent starting point for marker development. With the aid of the ~omics techniques, virtually everything is possible when it comes to clarifying the responsible genetic influencers. As with many things in life, if it is possible it does not necessary means it happens. The possibilities for research have enormously increased, the bottleneck for practical breeding programs will now be to keep up with the implementation of these techniques. Two of the crucial factors will become: accurate mass phenotyping of breeding material and setting up high throughput marker running platforms. The manpower and costs involved impose at this moment serious issues. However, with the advances in the field of phenotyping (for instance using computer coupled image analysis) and the ever lowering costs of sequencing and other molecular techniques in the end these problems might be overcome.

At that point potatoes with perfect quality will come within reach.

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Summary

In this thesis the results of the four-year project P5 are described. This project was carried out in the framework of the potato program of the Centre for BioSystems Genomics (www.cbsg.nl). The title of this project is “Unravelling the genetics of quality traits by a targeted QTL approach” and the intention was to gain understanding of the genetic principles underlying a series of different potato quality traits and the development of molecular markers linked to the traits of interest. These molecular markers can subsequently be used in breeding programs with the aim of producing potato varieties with improved quality.

To provide a framework in which the research of this thesis can be placed, a review of the current status of quality genetics in potato is provided in Chapter 1. Here the different potato tuber quality traits are described and different methodologies to assess them discussed.

The basis for the experimental work of this thesis is the diploid potato mapping population C x E, descending from the backcross between C (US-W5337.3) and E (77.2102.37). In the current research 249 full-sib descendants were selected and all clones were grown in multi-year repeats in the field (Wageningen, the Netherlands) during the normal potato growing season (April - September). Morphological observations were done and from a selection of clones starch and proteins were isolated from the harvested tubers of these field trails. An AFLP based linkage map was created, which was augmented with SSR and CAPS markers required for the genetic analysis.

Enzymatic discoloration (ED) of potato tubers was investigated in Chapter 2, in an attempt to unravel the underlying genetic factors. Both enzyme and substrate concentration have been reported to influence the degree of discoloration. Using the data for the degree of ED and levels of chlorogenic acid and tyrosine, Quantitative Trait Locus (QTL) analysis was performed. Three QTL’s for ED have been found on parental chromosomes C3, C8, E1 and E8. For chlorogenic acid a QTL has been identified on C2 and for tyrosine levels, a QTL has been detected on C8. None of the QTLs overlap, indicating the absence of genetic correlations between these components underlying ED, in contrast to earlier reports in literature. An obvious candidate gene for the QTL for ED on C8 is polyphenol oxidase (PPO) that was previously mapped on chromosome 8. With gene specific primers for PPO gene POT32 a CAPS marker was developed. Three different alleles (POT32-1, -2 and -3) could be discriminated, which showed high levels of sequence similarity. The segregating POT32 alleles were used to map the POT32 CAPS marker and QTL analysis was redone, showing that POT32 coincides accurately with the QTL peak. A

clear correlation between allele combinations and degree of discoloration was observed. In addition, analysis of POT32 gene expression in a subset of genotypes indicated a correlation between the level of gene expression and allele composition. On average, genotypes having two copies of allele 1 had both the highest degree of discoloration as well as the highest level of POT32 gene expression.

In Chapter 3, potato starch related traits and the underlying starch biosynthesis and degradation are investigated by a QTL analysis in combination with a candidate approach. The mapping population was assayed, during two consecutive years, for chipping colour, cold induced sweetening, starch content, starch granule size, starch gelling temperature, starch enthalpy, amylose content and degree of starch phosphorylation. QTL's were observed for all traits, except enthalpy. The QTL's were present on eight out of the twelve potato chromosomes. On chromosomes 2, 3, 5, 8, 9, 10 and 12, QTL's were identified that were consistent over the years. On a number of these chromosomes several clusters of co-localizing QTL's were observed, indicating common genetic factors for the different traits. Some of the combinations of co-segregating candidate genes and QTL's were previously reported, some co-localizations are reported for the first time. On chromosome 2 Soluble Starch Synthase 2 (SSSII) is mapped on the same position as QTL's for starch phosphorylation, starch gelling temperature and amylose content. α-Glucan-diwaterkinase (GWD) is co-localizing on chromosome 5 with QTL's for starch phosphorylation and cold sweetening. Furthermore the phosphorylases StPho1a and StPho2 coincide with QTL's for starch gelling, chipping colour and starch granule size on chromosome 2 and a QTL for starch phosphorylation on chromosome 9, respectively. The results suggest allelic variation acting on the genetics of the different traits. This variation is a starting point for further improvement of potato varieties by means of marker-assisted selection.

Metabolites are of utmost importance for the proper functioning and adaption of plants. Therefore, they also determine to a large degree the phenotypic traits of importance for human applications. However, a lot is still unknown about the metabolites itself and next to that plant metabolic content is still poorly understood with regard to its genetic basis. Here, in Chapter 4, we describe the genetic analysis of the metabolic variation present in tubers of the C x E population. Metabolic content was analysed through liquid chromatography-time of flight mass spectrometry (LC-QTOF MS). This resulted in a large set of mass peaks (14428 before and 3024 after background noise reduction), with clear qualitative and quantitative differences. Next to that, 876 mass signals were unique for the progeny population and could not be detected in either

of the parents. Clustering of the 3024 signals through a multivariate mass spectra reconstruction strategy revealed 233 reconstructed metabolites (ea centrotype) and 425 (14%) single non-clustered mass signals. Quantitative trait locus (QTL) analysis of the centrotypes revealed 803 metabolite-QTL's (mQTL's) with a significant LOD score, distributed over all 12 chromosomes. To investigate the contribution of the metabolome to phenotypic traits, the mQTL results were compared with QTL results for tuber flesh colour (raw and after-cooking) and total tuber protein content. For both traits co-localizing mQTL's were observed. The centrotypes linked to flesh colour were identified as being carotenoid derived compounds (Glucopyranoside) and carotenoids are amongst the most determining compounds of flesh colour. In relation with protein content, centrotypes were identified as glutamic acid (Glu) and valine (Val). Both are proteinogenic amino acids, compounds that are used as building blocks for proteins, therefore showing a clear relation to the trait total protein content. With this research we offer both new insights into the genetics of two potato quality traits as well as working proof of a method for unravelling the genetics of quality traits based on their underlying metabolic compounds.

In the final chapter, the general discussion, the results obtained in the preceding chapters are discussed and placed in the broader perspective of finding tools for breeding for quality traits in potato. In addition, prospects for the implementation of these results and an outlook for breeding for quality are presented.

Samenvatting

In dit proefschrift worden de resultaten van het vierjarig project P5 beschreven. Dit project is uitgevoerd binnen het Centre for Biosystems Genomics (www.cbsg.nl) en is getiteld "Opheldering van de genetica van kwaliteitseigenschappen in aardappel door middel van een gerichte QTL-analyse". Het doel van dit onderzoek was om de genetische principes achter een aantal verschillende kwaliteitseigenschappen van aardappel duidelijk te krijgen en tegelijkertijd moleculaire merkers te ontwikkelen voor een aantal van deze eigenschappen. De merkers kunnen vervolgens gebruikt worden voor selectiedoeleinden in de veredelingsprogramma's van de participerende veredelingsbedrijven met als einddoel de ontwikkeling van rassen met een verbeterde kwaliteit.

In Hoofdstuk 1 wordt het kader geschetst waar binnen het onderzoek van dit project geplaatst kan worden. De status van het onderzoek naar de verschillende kwaliteitseigenschappen van met name aardappelknollen wordt beschreven met een focus op de onderliggende genetica. Verder wordt ingegaan op de verschillende technieken en strategieën die gebruikt kunnen worden in de veredeling op kwaliteit.

De basis voor het experimentele werk, zoals beschreven in dit proefschrift, is de diploïde aardappelonderzoekspopulatie C x E welke het resultaat is van de terugkruising van de diploïde ouders C (US-W5337.3) en E (77.2102.37). De populatie bestaat uit 249 nakomelingen. Deze nakomelingen zijn vermeerderd in meerjarige experimenten op een proefveld in Wageningen gedurende het normale Nederlandse aardappelteeltseizoen (April – September). De aardappelknollenoogst van iedere nakomeling is onderworpen aan morfologische waarnemingen en van een selectie van de nakomelingen is de oogst gebruikt voor de isolatie van zetmeel, eiwitten en metabolieten. Een genetische kaart van de populatie, bestaande uit AFLP-merkers aangevuld met SSR- en CAPS- merkers, is gebruikt voor de genetische analyses.

Het onderzoeksonderwerp van Hoofdstuk 2 is de ontrafeling van de genetica achter enzymatische verkleuring van rauwe aardappelknollen. Voor deze eigenschap zijn volgens de literatuur twee factoren van belang, namelijk de betrokken enzymen en de concentratie van de substraten voor deze enzymen. Naast het niveau van verkleuring in de tijd is van de C x E populatie ook de hoeveelheid van de substraten chlorogeenzuur en tyrosine gemeten. De data van deze twee metingen is gebruikt voor een 'Quantitative Trait Locus' (QTL) analyse. Het resultaat van de analyses leverde drie QTLs op voor enzymatische verkleuring (op chromosomen E1, C3, C8/E8) en respectievelijk één QTL voor chlorogeenzuurgehalte op chromosoom C2 en

één QTL voor tyrosinegehalte op chromosoom C8. Geen van de gevonden QTL-regio's overlappen, waardoor de conclusie getrokken kan worden dat de eigenschappen niet beïnvloed worden door gemeenschappelijke genetische principes, maar dat er wel meerdere genen betrokken zijn bij deze kwaliteitseigenschappen.
Een voor de handliggend kandidaatgen voor verkleuring is het polyphenol oxidase-gen (PPO) dat in eerder onderzoek gelokaliseerd was op chromosoom 8. Gebruikmakend van primers ontwikkeld op de sequentie van het specifieke PPO-gen POT32 is een CAPS-merker gemaakt. Met deze merker konden in de CxE populatie drie verschillende allelen van POT32 worden onderscheiden, POT32-1, -2 en -3, welke een hoge mate van sequentie gelijkenis vertoonden. De segregerende allelen zijn gebruikt om de merker voor POT32 op de genetische kaart te plaatsen. Vervolgens is de QTL-analyse voor enzymatische verkleuring opnieuw gedaan met de verbeterde genetische kaart. Het resultaat van deze analyse is dat de QTL-piek voor verkleuring op chromosoom 8 exact boven de merker voor POT32 blijkt te liggen. Concluderend kan gesteld worden dat POT32 zeer waarschijnlijk één van de belangrijkste genetische factoren achter enzymatische verkleuring is. Deze hypothese wordt versterkt door de duidelijke correlatie tussen de verschillende allelcombinaties en de verschillende niveaus van verkleuring. Daarnaast blijkt er ook een correlatie te bestaan tussen het expressieniveau van het POT32-gen en de aanwezigheid, al dan niet afwezigheid, van bepaalde allelen. Gemiddeld genomen hebben genotypen met een homozygote aanwezigheid van allel 1 zowel het hoogste niveau van enzymatische verkleuring als ook het hoogste niveau van genexpressie.

In Hoofdstuk 3 worden aardappelzetmeel-gerelateerde eigenschappen en de onderliggende zetmeelsynthese en degradatiefactoren onderzocht. Het onderzoek richtte zich op een QTL-analyse van de betreffende eigenschap in combinatie met een kandidaatgen-aanpak. De onderzoekspopulatie van C x E is gefenotypeerd voor chipskleur, koude verzoeting, zetmeel-gehalte, zetmeelkorrelgrootte, zetmeelgellering-temperatuur, zetmeel-enthalpie, amylosegehalte en niveau van zetmeelfosforylering. Voor alle onderzochte eigenschappen zijn QTL's gevonden. De QTL's liggen verspreid over acht van de twaalf chromosomen van aardappel. Op chromosomen 2, 3, 5, 8, 9, 10 en 12 zijn QTLs gevonden die consistent zijn over de beide onderzochte jaren (2002 en 2003). Op een aantal van de chromosomen zijn clusters waar te nemen bestaande uit meerdere, overlappende QTL-regio's. Deze clusters duiden op gemeenschappelijke genetische componenten voor de verschillende eigenschappen. Sommige van deze clusters vallen samen met de locatie van een kandidaatgen, een aantal van de QTL-kandidaatgen combinaties zijn bekend uit de literatuur, andere zijn nieuw. Ter illustratie, op

chromosoom 2, ter hoogte van het oplosbaar zetmeel synthese II gen (SSSII), liggen QTLs voor zetmeelfosforylering, zetmeelgellering-temperatuur en amylosegehalte. De positie van het glucan water dikinase-gen (GWD), op chromosoom 5, valt samen met de QTLs voor zetmeelfosforylering en koude verzoeting. Ten slotte liggen op de posities van de zetmeelfosforyleringsgenen StPho1a (chromosoom 2) en StPho2a (chromosoom 9) QTLs voor zetmeelgelleringstemperatuur, chipskleur en zetmeelkorrelgrootte en respectievelijk zetmeel-fosforylering. De resultaten van dit onderzoek suggereren dat specifieke genetische variatie verantwoordelijk is voor meerdere, verschillende eigenschappen en dat deze eigenschappen op genetisch niveau dus verwant zijn. De onderliggende genetische variatie is het startpunt voor verdere verbetering van de bestaande aardappelrassen, gebruikmakend van de merkers uit dit onderzoek door middel van merkergestuurde selectie.

Voor het goed functioneren van planten in allerlei verschillende milieus zijn metabolieten van het grootste belang. Daardoor bepalen diezelfde metabolieten voor een groot gedeelte ook de fenotypische eigenschappen van planten die van belang zijn voor menselijke doeleinden. Echter, er is nog relatief erg weinig bekend over plantmetabolieten en daarmee samenhangend is er ook nog veel onbekend over de onderliggende genetische basis van zowel metabolietsynthese, -regulatie als ook -transport. In hoofdstuk 4 wordt de genetische analyse van de metabolische variatie in aardappels beschreven. De metabolietsamenstelling word geanalyseerd door middel van LC-QTOF massaspectrometrie. Het resultaat is een grote dataset van verschillende massapieken (14428 pieken voor, en 3024 na achtergrondreductie) met zowel duidelijke kwalitatieve als kwantitatieve kenmerken. Daarnaast zijn 876 van de 3024 massa pieken uniek voor de nakomelingen. Met andere woorden, deze pieken kunnen niet gedetecteerd worden in één van beide ouders. Clustering van de 3024 verschillende pieken door middel van een multivariate massaspectrum-reconstructie strategie resulteerde in 233 gereconstrueerde metabolieten (zogenaamde centrotypen) en 425 (14%) enkele, niet-geclusterde massapieken. Directe QTL-analyse van de centrotype dataset resulteert in 803 metaboliet-QTLs (mQTLs) met een significante LOD-score, en verdeeld over alle 12 aardappelchromosomen. Om te onderzoeken wat de bijdrage van de metabolieten is aan de verschillende fenotypische eigenschappen van aardappel, zijn de mQTL-resultaten vergeleken met de QTL-resultaten van de eigenschappen vleeskleur (rauw en na koken) en totaal eiwitgehalte van de knol. Voor beide eigenschappen zijn er clusters te vinden van fenotypische QTLs met mQTLs. De centrotypes die gekoppeld kunnen worden aan vleeskleur zijn geïdentificeerd als zijnde carotenoïde-gerelateerde stoffen (glycopyranosiden). Algemeen bekend is dat het carotenoïdengehalte sterk gecorreleerd

is aan vleeskleur. De aminozuren glutaminezuur (Glu) en valine (Val) zijn geïdentificeerd in relatie tot totaal eiwitgehalte. Beide aminozuren zijn essentiële bouwstenen voor eiwitten: waarschijnlijk is er een directe relatie tussen een hoog gehalte aan deze beide aminozuren en een hoog gehalte aan eiwit. Met deze onderzoeksresultaten bieden we nieuwe inzichten in de genetica van twee aardappeleigenschappen en tegelijkertijd tonen we een methode om de genetica van kwaliteitseigenschappen op te helderen via de onderliggende metabolieten.

In het laatste hoofdstuk, de algemene discussie, worden de resultaten uit de eerdere hoofdstukken bediscussieerd en in een breder perspectief geplaatst wat betreft de ontwikkeling van methodes van veredeling op kwaliteit in aardappel. Ten slotte worden de vooruitzichten met betrekking tot implementatie van de resultaten en veredeling voor kwaliteit in het algemeen gepresenteerd.

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In my case, I think I can safely say: FINALLY!!!! Finally, I have reached the end of the long journey called “my PhD”. A journey that in the end took me exactly twice the amount of time as planned at the beginning in 2003. This long period not only shaped my professional skills but also developed me into the person that I am today. Not only the research, the travels and the experiences shaped me, but also the interaction with all the different people I met on this travel. Therefore, I would hereby like to acknowledge everybody who supported me over the years, each and everyone in their own way.

The start of my PhD project was almost a logical consequence of the work I did earlier during my MSc thesis and shortly after my graduation. Both projects were on potato and as the Dutch saying goes, “driemaal is scheepsrecht”. Even though it seemed logical to me I still had to be accepted for the job. On this point I would like to thank my promoter Prof. Dr. Richard Visser and my co-promoter Dr. Christian Bachem for giving me the opportunity to do my PhD in their department and research group respectively. Richard, over the years you have been a very stable factor in all the matters surrounding my research project and thesis. Even though your schedule got considerably busier over the years, you always managed to find some time for me when I needed your advise or opinion, no matter if it was a week day, a Saturday afternoon or a Sunday evening. Your clear perspective on the different topics and the fact that you’ve supported me through all the critical moments till the very end are highly appreciated.

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Through the years and different office moves, I have had quite a few room mates. In chronological order, Dirk Jan, Karin, Richard, Bjorn, Asun, Nicolas and Antoine, thank you for the pleasant atmosphere you've all created in the different offices!
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By the time this thesis is ready, I've spend as much time at plant breeding as at Enza. Therefore also thanks to my colleagues from Enza, and especially the members of the Dutch cucumber breeding team for their caring over the years and their support and interest where needed. Special thanks to Antonio Sances Lopez who gave me the opportunity to create some room in the busy cucumber work in order to keep on working on my thesis. I am not sure if I would have succeeded otherwise, thank you! Furthermore Jan Draaistra, who from his own experience knew what I was going through, your support and advise are well appreciated. Finally Joep Lambalk, the fact that my director every now and then asked about the progress gave me the "kick under the ass" that I needed to keep going.

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pleasant verbal "fights", fortunately when you shout "Jeroen!!!!" via Skype I can still hear it clearly. I am happy that two of my friends are also my paranymphs, Björn d'Hoop and Nicolas "Nico" Champouret. Although the first to start I am now the last to finish, but by having the two of you as my backup the circle is complete. We shared a lot of things together: fun (travels, weddings, births, parties and beers), good meals (biefstuk/frites, raclette and pasta and wine), hard work (in the gym, in new houses and at work) and some serious discussions at the "Vlaam". Even though now we don't live in the same country anymore or we can't discuss about work as we work for competing companies, let's make sure we keep in touch!

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Jeroen

Curriculum Vitae

Jeroen Sebastiaan Werij was born on the 9th of August 1980 in Harderwijk, the Netherlands. At high school (VWO) in Amersfoort, he developed a fascination for genetics and more specifically plant genetics. As a consequence, he started his BSc in Plant breeding and crop protection (T15) at the Wageningen Agricultural University in 1998. In January 2003 he finished his MSc with a specialization in molecular plant breeding at Wageningen University. After finishing his studies he started in May 2003 as researcher at the laboratory of plant breeding, Wageningen University. The topic of the work was mainly the isolation of BAC DNA of the potato BAC library later on used for the sequencing of the potato genome. In November 2003 he stopped this job in order to start a PhD project at the same laboratory under the supervision of Dr. Christian Bachem and Prof. Dr. Richard Visser. The subject of this project was the genetic analysis of tuber quality traits in potato. The results of this research project are presented in this PhD thesis. Since January 2008 he is working as cucumber breeder at Enza Zaden, focussing on the Long European Type (LET) for heated crops in NW-Europe and N-America.

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Education Certificate

Education Statement of the Graduate School Experimental Plant Sciences	
Issued to: Jeroen S. Werij	
Date: 18 November 2011	
Group: Plant Breeding, Wageningen University and Research centre	
1) Start-up phase	date
► **First presentation of your project**	
Unraveling the genetics of quality traits in potato by a targeted QTL approach	Mar 08, 2004
► **Writing or rewriting a project proposal**	
► **Writing a review or book chapter**	
► **MSc courses**	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*1,5 credits**
2) Scientific Exposure	date
► **EPS PhD student days**	
EPS PhD student Day, University of Amsterdam	Jun 03, 2004
EPS PhD student Day, Radboud University Nijmegen	Jun 02, 2005
EPS PhD Student Day, Wageningen University	Sep 19, 2006
► **EPS theme symposia**	
Theme 4 'Genome Plasticity', Radboud University Nijmegen	Dec 10, 2003
Theme 4 'Genome Plasticity', Wageningen University	Dec 09, 2004
Theme 4 'Genome Plasticity', Radboud University Nijmegen	Dec 08, 2006
Theme 3 'Metabolism and Adaptation', Wageningen University	Nov 06, 2007
► **NWO Lunteren days and other National Platforms**	
ALW Meeting Lunteren 2004	Apr 05-06,2004
ALW Meeting Lunteren 2005	Apr 04-05, 2005
ALW Meeting Lunteren 2006	Apr 03-04, 2006
ALW Meeting Lunteren 2007	Apr 02-03, 2007
► **Seminars (series), workshops and symposia**	
Flying seminars (4x); Clark, Ecker, Benfey, Yano	2004 - 2008
CBSG Patenting workshop (afternoon)	May 23, 2005
Prof. Dr. Kazuto Iwama, Hokkaido University, Japan	Nov 17, 2006
Dr. Roger Hellens, Hort Research, New Zealand	Feb 14, 2007
Dr. George Harrigan, Monsanto, USA	Jun 08, 2007
Prof. Kaiyun Xie, IVF-CAAS / CIP-China Office, Beijing, China	Jun 12 2007
► **Seminar plus**	
► **International symposia and congresses**	
Meeting of EAPR: Section Breeding & Varietal Assessment and Eucarpia: Potato (Carlow, Ireland)	Nov 20-22, 2006
4th Solanaceae Genome Workshop, Jeju, South-Korea	Sep 09-13, 2007
► **Presentations**	
Oral presentation at the CBSG meeting (twice a year)	2004-2007
Poster presentation Joint Section Meeting EAPR, Carlow (Ireland)	Nov 20-22, 2006
Oral presentation at Plant Breeding Seminar Series	Feb 19, 2007
Poster presentation 4th Solanaceae Genome Workshop, Jeju, South-Korea	Sep 09-13, 2007
Oral presentation 4th Solanaceae Genome Workshop, Jeju, South-Korea	Sep 09-13, 2007
► **IAB interview**	Sep 18, 2006
► **Excursions**	
Subtotal Scientific Exposure	*20,3 credits**
3) In-Depth Studies	date
► **EPS courses or other PhD courses**	
EPS course Molecular phylogenies: reconstruction & interpretation	Oct 16-20, 2006
Course Basic Statistics	Dec 18-22, 2006
► **Journal club**	
Literature discussion at Plant Breeding group	2004-2008
► **Individual research training**	
Learning SSR marker techniques at Plant Research International, Wageningen	Apr 25-May 27, 2005
Subtotal In-Depth Studies	*9,0 credits**
4) Personal development	date
► **Skill training courses**	
Scientific Publishing	Oct 13, 2005
Scientific Writing	Feb-Apr, 2006
► **Organisation of PhD students day, course or conference**	
► **Membership of Board, Committee or PhD council**	
Membership EPS student Council	2006-2007
Subtotal Personal Development	*3,5 credits**
TOTAL NUMBER OF CREDIT POINTS*	**34,3**
Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits	
** A credit represents a normative study load of 28 hours of study.*	

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