Transcriptomic analysis of potato tuber development and tuber quality traits using microarray technology

In search of candidate genes

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Transcriptomic analysis of potato tuber development and tuber quality traits using microarray technology - *In search of candidate genes*

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With references – with summaries in English and Dutch

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Chapter 1

General Introduction

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The potato crop

The potato, *Solanum tuberosum* L. originates from the Andes region in South America and was brought to Europe in the late 16th century. From here, the potato plant has been extensively cultivated and spread all over the world as a major food crop. Potato plants have the capacity to propagate vegetatively by producing underground tubers (Figure 1). These tubers are actually modified thickened stems that are formed during the growing season. During the process of tuber formation and subsequent tuber enlargement, the growing tubers act as strong sinks for photo assimilates transported down from the leaves. Fully matured tubers are therefore a rich source of energy, with starch constituting around 80% of tuber dry weight, with a high content of quality proteins and vitamin C. Potato has become the fourth major food crop with an annual production of around 326 million tons (www.fao.org). Potato tubers are mainly grown for either human consumption, usage in cattle feed, production of seed potatoes or for the isolation of potato starch for use in a wide range of industrial applications. This diverse use of potato tubers and the different climatic conditions in parts of the world where potatoes are grown require the availability of a large collection of potato cultivars optimized for their respective application or growing conditions. For this reason the processes related to potato tuber formation and tuber quality traits have been intensively studied. Moreover, the potato plant serves as a model plant for studying underground sink organ formation and therefore further understanding of the processes related to tuber formation and tuber quality traits serves not only an economical but also a fundamental interest.

Figure 1: Photograph of underground stolon and tuber growth.

Potato tuber formation and morphology

The formation and growth of a potato tuber is a complex process which can be divided into separate physiological events. When a growing potato plant reaches a certain maturity or size, underground stem like structures, called stolons, are formed. A stolon may be defined as a lateral shoot, showing negligible leaf expansion, possessing a hook-shaped tip and growing diageotropically (Booth, 1963). Under environmentally favourable conditions, potato plants are induced to tuberize through the production of a graft transmissible signal that is transported basipetally from the leaves to the growing stolon tip where it promotes tuberization (Gregory, 1956; Jackson *et al.*, 1998). The nature of this transmissible signal is still unknown but is most likely based on a mixture of both inducing and inhibiting types of signals (Ewing and Struik, 1992; Jackson, 1999; Martinez-Garcia *et al.*, 2001). When plants are induced to tuberize, longitudinal stolon growth is inhibited followed by subapical swelling of the stolon tip (Cutter, 1978; Peterson *et al.*, 1985). In an *in vitro* tuberization experiment it was shown that the level of the plant hormone gibberellic acid A1 (GA_1) dramatically decrease in the stolon tip prior to first visible swelling (Xu *et al.*, 1998a).

The observed initial swelling is a result of increased radial cell expansion shortly accompanied by longitudinal cell division in the pith and cortex (Sanz *et al.*, 1996; Xu *et al.*, 1998b). When tubers reach a diameter of around 0.8 cm, longitudinal cell division stops but randomly orientated cell division and cell enlargement occurs in the perimedullary region until tubers reach their final diameter (Xu *et al.*, 1998b). The perimedullary region forms the major portion of the mature tuber and consists of storage parenchyma cells and vascular tissues (Figure 2). During tuber growth, starch, storage proteins and a wide range of metabolites accumulate within the tuber. Final potato tuber size, morphology and content, together representing different aspects of tuber quality, are largely dependent on the potato genotype and environmental conditions.

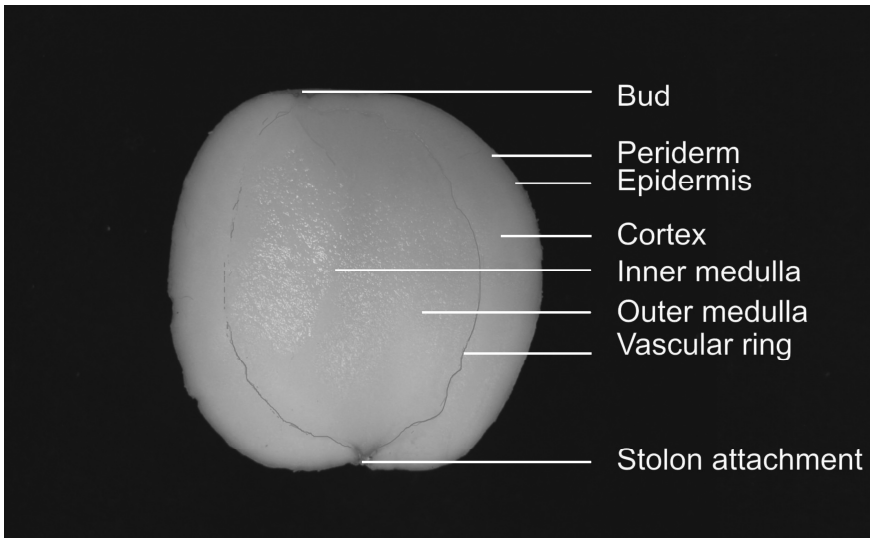

Figure 2: Cross section of mature potato tuber with the different tissues indicated.

Factors influencing the process of tuber formation

Photoperiod

Potato tuber induction and growth is regulated trough the interplay of environmental signals, plant hormones and signalling molecules. A major factor controlling the time point of tuber induction is day length. In general, tuberization is promoted by long nights (i.e. short days) with the degree of response largely dependent on genotype and physiological age of the plant (Ewing and Struik, 1992). In plants requiring SD conditions in order to tuberize, interrupting a long dark period with a night break of red light inhibits tuberization and this inhibition can be partially reversed by a subsequent treatment with far-red light (Jackson, 1999). Photoperiod perception takes place in the leaves through the action of photoreceptors including phytochromes. Specifically the action of phytochrome B (PHYB) has been linked to the formation of the tuber inducing signal in potato. Anti-sense *phyB* plants lost their photoperiod-dependent tuberization and tuberized equally well under both long day (LD) and short day (SD) conditions (Jackson *et al.*, 1996). Grafting experiments showed that PHYB is involved in the production of a transmissible inhibitor of tuberization in the aerial parts of the plant, since wild type plant stocks were induced to tuberize in LD when grafted with a scion from an anti-*phyB* plant (Jackson *et al.*, 1998). Although much effort has been made in attempting to unravel the mechanisms underlying photoperiod dependent tuberization, modern day cultivars have lost most of their strict photoperiodic regulation and no longer require short day (SD) conditions in order to form tubers.

Plant Hormones

Plant hormones, particularly gibberellic acid (GA) have long been implicated to play a key role in regulating potato stolon growth and tuber initiation (Booth, 1963; Kumar and Wareing, 1974; Ewing, 1987). GAs are cyclic diterpenoid hormones that regulate many plant growth and developmental processes including germination, stem growth, flowering, fruit and tuber development (Lange, 1998). Applications of biologically active GAs or inhibitors of GA biosynthesis have shown to either delay or promote tuber formation under tuber inducing conditions (Vreugdenhil and Struik, 1989; Jackson and Prat, 1996). GA levels are reduced in potato plants when transferred to short-day conditions, indicating a direct link between photoperiod and GA levels in controlling tuber formation. Furthermore, over-expression of a potato GA20-oxidase gene, synthesizing a major precursor of bioactive GAs (GA_{20}), results in taller plants with delayed tuber formation under inducing conditions (Carrera *et al.*, 2000). Xu *et al*., (1998a) quantified endogenous GA levels during various stages of stolon elongation and tuber formation. They found that GA_1 levels were high during the longitudinal elongation of stolons and dramatically decreased prior to first visible swelling. Shibaoka *et al*., (1993), showed that GA_3 can regulate the orientation of the microtubules and micro fibrils in plant cells. A reduction of GA, as found in the stolon tip, may therefore cause the reorientation of the plane of cell expansion and cell division resulting

in subapical swelling of the stolon (Fujino *et al.*, 1995; Xu *et al.*, 1998a). Whether biologically active GAs or the precursors themselves are part of a transmissible signal or play a role in the production, sensitivity or transport of tuber promoting or inhibiting signals within the plant remains to be resolved. Moreover, the regulatory mechanisms by which GA levels in the stolon are reduced prior to tuber induction are still unknown.

Other plant hormones like abscisic acid (ABA), indole acetic acid (IAA), cytokinin and jasmonic acid (JA) are currently thought to play only minor roles in regulating tuber formation. These hormones have exhibited either promoting or inhibiting effects on tuber formation relative to the concentrations applied (Koda and Okazawa, 1983b; Vreugdenhil and Struik, 1989; Xu *et al.*, 1998a). Endogenous hormone levels or ratios often vary during tuber organogenesis and may have functions in promoting cell expansion and cell divisions or exert their effect principally by antagonizing or strengthening the effect of other hormones (Koda and Okazawa, 1983a; Vreugdenhil and Struik, 1989; Xu *et al.*, 1998a; Abdala *et al.*, 2002; Cenzano *et al.*, 2003). The recent findings of auxins controlling GA metabolism in pea shoots may lead to a re-evaluation of the regulatory functions of particularly auxin in relation to GA levels in potato tuber development.

Sucrose

The *in vitro* potato tuberization system, implemented in many tuber development studies (Hendriks *et al.*, 1991; Appeldoorn *et al.*, 1997), is characterized by the highly synchronous formation of microtubers from single nodal stem cuttings on medium with high sucrose (8% w/v). The frequency of tuberization is relative to the amount of sucrose in the medium suggesting an inducing role for sucrose in tuber formation. Xu *et al.*, (1998a) suggested a negative effect of sucrose on GA-levels within the stolon tip thereby promoting tuber formation. Furthermore, sucrose synthesis and export from the leaves is affected by photoperiod, indicating sucrose may act as a signalling molecule for tuber induction. Reduction of sucrose transport results in a reduction in tuber formation and tuber yield (Riesmeier *et al.*, 1994; Kuhn *et al.*, 2003). It is however, difficult to resolve the importance of sucrose in tuber induction *in vivo* plants since a reduction in sucrose availability leads to a reduced development of other sink organs and thus an altered metabolic status of the plant (Riesmeier *et al.*, 1994).

Other Factors

Besides endogenous factors like plant hormones and sugars controlling tuber initiation and growth, exogenous signals can have a large impact on tuber formation. For example, application of high nitrogen levels to the roots can inhibit tuber formation. Removal of nitrogen supply under tuber inducing conditions results in tuber formation within 5 to 6 days (Krauss, 1995). Interestingly, reducing nitrogen supply under non-inducing conditions, such as LD, will not result in tuber formation, indicating that nitrogen is probably not involved in

the induction of tuberization but that it is able to repress tuber formation once it is induced (Krauss, 1995).

Like nitrogen levels, high temperatures are inhibitory for tuber formation and affect the production of the tuber inducing signal (Bodlaender, 1963). Furthermore, hot weather can result in re-growth of a tuber where stolon growth is initiated from the tuber in a process called heat sprouting (Jackson, 1999). Similar to elevated nitrogen levels, high temperatures may mediate their inhibitory effect through increased GA levels (Jackson, 1999). It is important to understand that potato tuber formation is a flexible process that can be influenced through a wide variety of signals that act independently or in combination (Figure 3). Under changing conditions potato plants have the capability to switch from a non-inducing to a tuber inducing state and *vice versa*. Several of these factors appear to mediate their effect through regulating GA levels within the plant. Since GA alone cannot overcome the strict need for SD conditions in certain photoperiod-dependent species, this indicates the existence of at least two major pathways controlling tuber formation; a photoperiodic-dependent pathway and a GA-dependent pathway (Martinez-Garcia *et al.*, 2001).

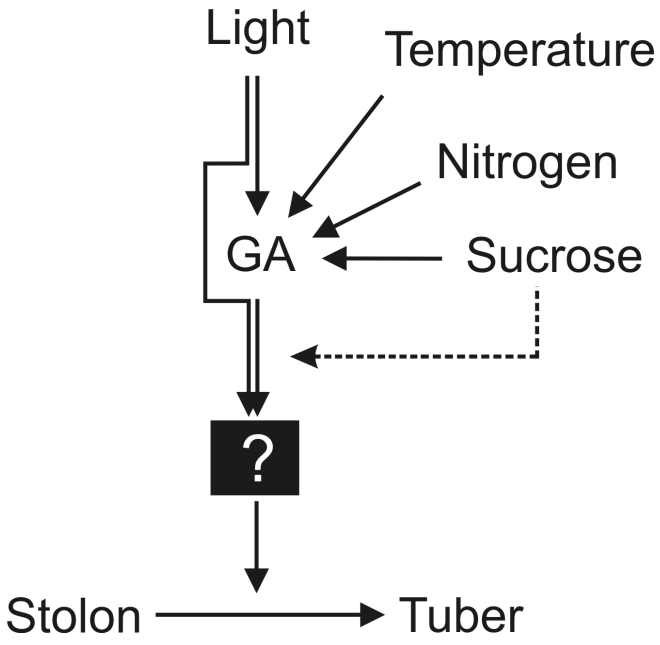

Figure 3: Schematic overview of factors influencing *in vivo* potato tuberization.

Gene expression associated with potato tuber development and tuber growth

Both environmental factors (light, temperature, nitrogen) and endogenous factors (hormones, sugar) can affect potato tuber formation as described in the previous section. Researchers therefore, are interested in identifying the genes that are regulated by these factors in an attempt to establish a coherent sequence of events leading to the formation of a tuber. More specifically, the identification of the so called 'tuber identity genes', the genes inducing the onset of tuber formation or genes controlling a specific tuber quality

characteristic, are of great interest. Early studies of gene expression during potato tuber formation yielded differential expression of several patatin and proteinase inhibitors genes which are highly expressed storage proteins but obviously not the inducers of potato tuber formation (Prat *et al.*, 1990). More recent studies aimed at unravelling the mechanism controlling potato tuberization, has led to the identification of genes involved in both the photoperiod-dependent and GA-dependent tuber inducing pathway: *phyB* (Jackson *et al.*, 1996), *phor1* (Amador *et al.*, 2001), *stgan* (Bachem *et al.*, 2001), *CONSTANS-like* (Martinez-Garcia *et al.*, 2002), *poth1* (Rosin *et al.*, 2003), and s*tbel5* (Chen *et al.*, 2003). In addition, a group of genes has been isolated which exhibited differential expression during tuberization. These were implicated in playing a regulatory role in tuber development although their direct involvement remains ambiguous (Kang and Hannapel, 1996; Kolomiets *et al.*, 2001; Raices *et al.*, 2003). A more high-throughput approach, implementing cDNA-AFLP, provided a large set of differentially expressed transcripts related to early tuber development (Bachem *et al.*, 1996). Most genes mentioned above have been independently linked to tuber development although their precise functions in the process remains unclear.

After initial tuber onset, the young tuber grows rapidly through continued cell division and expansion in the perimedullary region that acts as a strong sink for photo assimilates primarily in the form of sucrose (Burton, 1966). During tuber initiation there is a switch in the primary pathway of sucrose import from apoplastic to symplastic sucrose unloading (Appeldoorn *et al.*, 1997; Viola *et al.*, 2001). As a result, the increased sucrose availability and subsequent sucrose cleavage products promotes the expression of a large set of genes involved in the production of starch or other storage components in the actively growing organ (Koch, 1996; Geigenberger, 2003). Besides starch and other storage proteins, the accumulation of metabolites like amino acids, flavonoids and other health related compounds requires the existence of a highly complex network of genetic regulation. Little is known about the genes regulating secondary metabolism in the potato tuber during development and subsequent tuber growth stages whilst in recent years the presence of secondary metabolites in food crops with regards to their nutritive values has become increasingly important (Verpoorte and Memelink, 2002). With the availability of large potato ESTs libraries (Ronning *et al.*, 2003) and the continuing development of functional genomic and bioinformatics tools, the identification of regulatory genes in all mentioned areas should become more efficient. Novel candidates for potato tuberization or quality traits are continuously required in order to achieve progress in the unravelling of the complex regulatory mechanisms underlying potato tuber growth and determination of tuber quality traits.

Functional genomics in potato

In the last decade novel molecular biology tools have rapidly evolved to become high throughput methods for analyzing a large number of samples in a short time frame and cost-effective manner. The different platforms or profiling techniques can be divided on the basis of their targeted substrates and include, gene expression (transcriptomics), proteins

(proteomics) and metabolites (metabolomics). Functional genomics is a general approach toward understanding how the genes of an organism work together by assigning new functions to unknown genes (Holtorf *et al.*, 2002). The elucidation of gene function based on profiling techniques can be greatly enhanced by the integration or coupling of data of the different platforms (Fiehn *et al.*, 2000; Fiehn *et al.*, 2001). In most research however, the different technologies are still primarily linked to plant diversity with respect to different plant morphology, disease susceptibility or quality traits (phenomics).

There are several large scale transcript profiling techniques available to the molecular biologist including; cDNA-AFLP (Bachem *et al.*, 1996), serial analysis of gene expression (SAGE; Velculescu *et al.*, 1995), massive parallel signature sequencing (MPSS; Brenner *et al.*, 2000) and the most successful of all, microarray technology (Schena *et al.*, 1995; Duggan *et al.*, 1999). The great advantage of microarray-based expression analysis is its sensitivity and the large number of genes, known or unknown, which can be monitored at the same time (Schena, 1996). The genes (cDNA or oligo's) are immobilized on a solid support, usually a membrane or coated glass slide, and hybridized with dye-labelled RNA pools of two or more samples allowing detection of differentially expressed genes across experiments (Figure 4). The success of a microarray profiling experiment largely depends on the array design, experimental setup, sampling and data analysis.

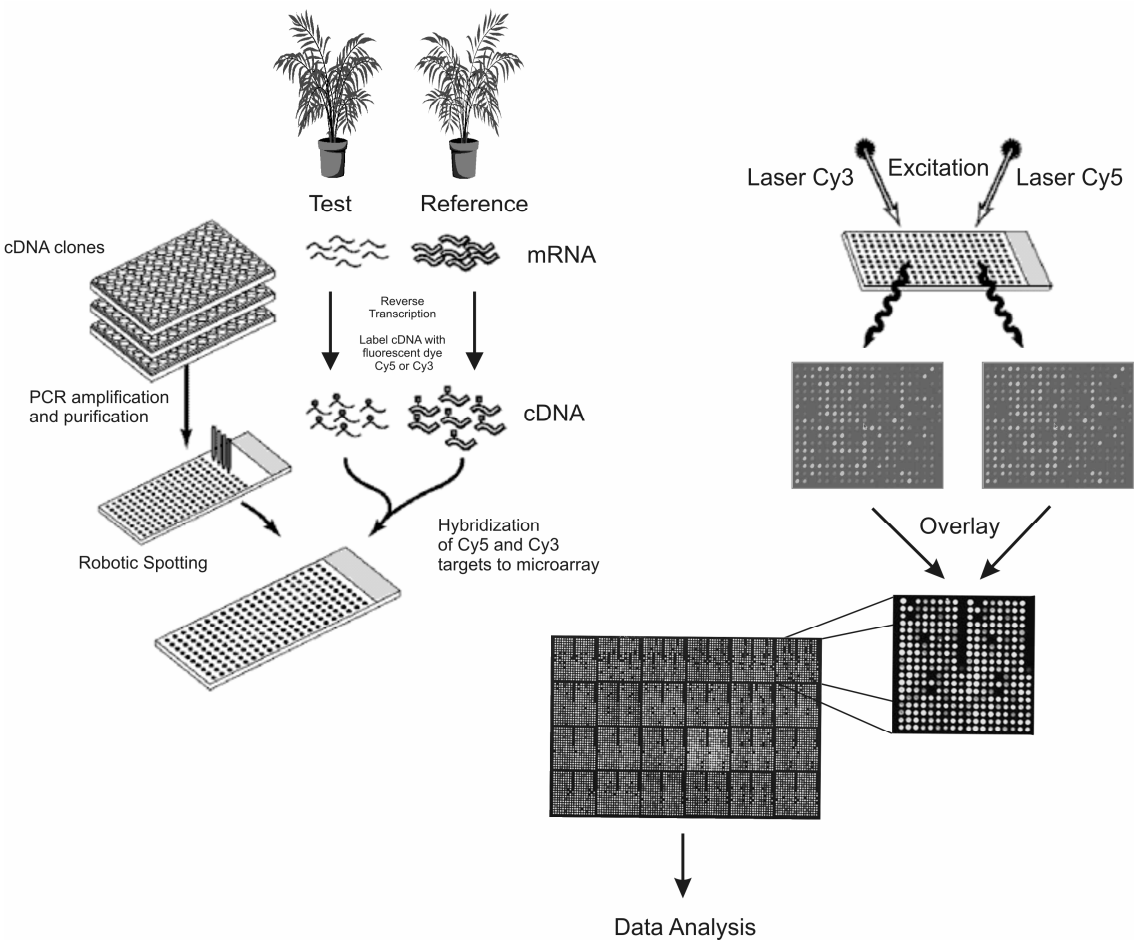

Figure 4: Overview of cDNA-microarray technology using two-colour fluorescence hybridization.

There are many excellent reviews discussing these aspects in greater detail (Schena, 1996; van Hal *et al.*, 2000; Finkelstein *et al.*, 2002; Clarke and Zhu, 2006). cDNA clone selection for microarray gene expression analysis can be either random or targeted via library subtractions or based on functional homologies (dedicated arrays). EST sequencing projects in potato (Crookshanks *et al.*, 2001; Ronning *et al.*, 2003) has provided the scientific community with an excellent resource for finding candidate genes and clones for expression analysis and the first potato cDNA microarrays based on a random selection of clones are now commercially available (http://www.tigr.org/tdb/potato). Other transcript profiling studies in potato include, *in vitro* tuberization using cDNA-AFLP (Bachem *et al.*, 1996) and expression profiling of mature tubers using SAGE (Nielsen *et al.*, 2005) and have provided a number of interesting candidate genes.

Genetical analysis in potato

Although we have clearly entered the decade of genomic research with novel technologies rapidly increasing the efficiency of studying entire biological systems, they often fail to unravel the complex nature of certain developmental processes or quality traits and the power of genetics becomes apparent. Potato tuber quality traits for example are for a large part dependent on the allelic composition of the genotype analyzed, but are also dependent on field growing conditions and environmental factors of that particular harvest year. Classical plant breeding relies on the genetic variation and the selection for favourable genotypes in the progeny after crossing, resulting in improved cultivars. The cultivated potato grown is a heterozygous vegetative propagated tetraploid species with multiple allelism (van de Wal *et al.*, 2001). Potato is an outbreeder and suffers from inbreeding depression when self-fertilized. Consequently, the cultivated potato itself is a highly heterozygous, autotetraploid species (2n=4x=48) making genetic studies of specific loci complex. Therefore, most genetic studies in potato are done at the diploid level.

There have been several genetic maps constructed in potato implementing different molecular marker technologies, based on sequence polymorphisms in either the whole genome, RFLP (Tanksley *et al.*, 1992), AFLP (van Eck *et al.*, 1995) or transcriptome, cDNA-AFLP (Brugmans *et al.*, 2002). Recently, an Ultra-High Density (UHD) potato AFLP map was constructed from a heterozygous diploid potato population in an attempt to saturate the genome with markers (Isidore *et al.*, 2003; http://www.dpw.wau.nl/uhd/). The map positions of the generated molecular markers can be used in marker assisted selection (MAS) for breeding purposes or for the cloning of candidate genes responsible for the determination of specific traits.

In a segregating population of potato, complex traits of significance in crop physiology and yield, exhibit a quantitative inheritance. Quantitative traits show phenotypic variation as a result of the combined allelic effect of genes and their interaction with environmental conditions. The genetic loci controlling quantitative traits are known as quantitative trait loci (QTL). QTL mapping is based on the association between phenotypic differences of the trait of interest and molecular markers present in linkage groups in genetic

maps. QTL analysis in potato has focussed for a large part on quantitative disease resistance, reviewed in Gebhardt and Valkonen (2001), and tuber quality traits including tuber shape (van Eck *et al.*, 1994), cold-sweetening (van Eck *et al.*, 1994; Menendez *et al.*, 2002), tuber dormancy (Freyre *et al.*, 1994) and tuber starch content (Schäfer-Pregl *et al.*, 1998). The molecular basis for the observed variation with regards to a specific QTL can be explained by a wide range of small or large sequence polymorphisms including; single nucleotide polymorphism (SNP), insertions, deletions and others that potentially could lead to altered gene function or regulation. Subsequent identification of candidate genes responsible for the genetic control of QTLs not only enhances our understanding of plant adaptation but may also aid future marker assisted selection strategies.

Genetical genomics and candidate genes

QTL cloning in plants is predominantly based on positional cloning approaches for which the success rate is dependent on several factors including the requirement of a high mapping resolution within a chromosomal interval to reduce the number of potential candidate genes (Salvi and Tuberosa, 2005). The use of functional genomic tools can contribute to several aspects of QTL analysis and cloning. Differential gene expression within a population can be considered as a quantitative trait that can result in the mapping of gene expression as a proper QTL or so-called eQTL (Schadt *et al.*, 2003). The combination of expression profiling, of either transcripts, protein or metabolites, with genetics has been referred to as 'genetical genomics' and is expected to greatly advance our capabilities to resolve metabolic, regulatory and developmental pathways (Jansen and Nap, 2001; Li and Burmeister, 2005). The use of microarray technology for accurately scoring of differential gene expression within large populations has resulted in the identification of novel candidate genes underlying specific traits of interest (Brem *et al.*, 2002; Wayne and McIntyre, 2002; Schadt *et al.*, 2003; Kirst *et al.*, 2004). In potato, the currently ongoing large scale EST sequencing projects and the availability of segregating potato populations, allows the potato researcher to explore the added value of combining large scale genomic tools with genetics.

Scope of this Thesis

In order to understand how potato plants regulate the formation of potato tubers and determine the different tuber quality traits, we need to identify and characterize the genes that are involved in regulating these processes. With the arrival of high throughput functional genomics tools like microarray technology, studying gene expression during potato tuber development and in relation to quality traits on larger scale, becomes feasible. To find novel candidate genes involved in potato tuber formation or quality traits, a dedicated cDNA-microarray was constructed in order to study gene expression during potato tuber formation and growth. Additionally, the constructed dedicated array was used in a screening of a diploid potato population, segregating for a number of important tuber quality characteristics, in

order to combine the power of functional genomics and genetics ('genetical genomics') for the identification of candidate genes.

In *chapter 2* the construction of the dedicated potato tuber life cycle cDNA-microarray is presented together with the expression data of genes during eight early tuber developmental stages. Common gene expression profiles are identified and novel candidate genes are discussed in relation to their role in the process of tuber formation.

In *chapter 3*, a candidate gene involved in the regulation of potato tuber development is further analyzed. A potato GA 2oxidase (*StGA2ox1*) gene is strongly up-regulated prior to tuber formation and is shown to play a role in regulating GA levels within the developing tuber.

In *chapter 4*, data is presented of the characterization of a gene with homology to members of the Aux/IAA gene family and is strongly down regulated during tuber formation (*StIAA2*). Transgenic plants with reduced expression of *StIAA2* show distinctive phenotypes that can be ascribed to altered auxin signalling throughout the plant.

In *chapter 5*, we present the analysis of gene expression related to potato tuber quality traits using a bulked segregant analysis approach. A proof of concept of this approach is presented based on the identification of a candidate gene involved in difference in texture after cooking characteristics (*StTLRP*).

In *chapter 6*, the results obtained in the previous chapters are discussed in light of the identified candidate genes and their roles in potato tuber development or tuber or potato tuber cooking quality. Furthermore, the potential power of integrating genomic tools and genetics is described for potato with the focus on applications for finding candidate genes in the future.

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Chapter 2

Tuber on a Chip: Differential gene expression during potato tuber development

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Abstract

Potato tuber development has proven to be a valuable model system for studying underground sink organ formation. Research on this topic has led to the identification of many genes involved in this complex process and has aided in the unravelling of the mechanisms underlying starch synthesis. However, less attention has been paid to understand the biochemical pathways of other important metabolites or changing metabolic fluxes occurring during potato tuber development. In this paper, we describe the construction of a potato cDNA-microarray, specifically designed for genes involved in processes related to tuber development and tuber quality traits. We present expression profiles of 1315 cDNAs during tuber development where the predominant profiles were strong up- and down-regulation. Gene expression profiles showing transient increases or decreases were less abundantly represented and followed more moderate changes, mainly during tuber initiation. Besides the confirmation of gene expression patterns during tuber development, many novel differentially expressed genes were identified and are considered candidate genes for direct involvement in potato tuber development. A detailed analysis of starch metabolism genes provided a unique overview of expression changes during tuber development. Characteristic expression profiles were often clearly different between gene family members. A link between differential gene expression during tuber development and potato tissue specificity is described. This dataset provides a firm basis for the identification of key regulatory genes in a number of metabolic pathways that may provide researchers with new tools to achieve breeding goals for use in industrial applications.

Introduction

In the last decade, potato has become the model system for studying the development of underground sink organs. The induction and formation of a potato tuber from underground stem-like structures, the stolons, follows a developmental program that requires the coordinate regulation of many metabolic pathways. Multiple signalling pathways and environmental factors have been described in relation to potato tuber development (Jackson, 1999; Fernie and Willmitzer, 2001). Photoperiodic control of potato tuberization, with long dark periods promoting tuberization, involves the regulation of a graft-transmissible tuberization signal most likely consisting of a combination of inhibiting and tuber inducing stimuli (Jackson *et al.*, 1998; Jackson, 1999; Martinez-Garcia *et al.*, 2001). The actions of plant hormones and in particular gibberellins (GAs), have long been implicated in the regulation of tuber development (Ewing, 1987; Vreugdenhil and Struik, 1989). It has been clearly shown that a reduction of active GA coincides with potato tuberization (Xu *et al.*, 1998a) and inhibitors of GA biosynthesis can promote tuberization (Jackson and Prat, 1996). Furthermore, lowered transcript levels of a GA biosynthesis gene, GA 20-oxidase, accelerated tuberization under inducing conditions (Carrera *et al.*, 2000). Research aimed at unravelling the mechanism controlling potato tuberization, has led to the identification of genes involved in both the photoperiod-dependent and GA-dependent pathway: *phyB*

(Jackson *et al.*, 1996), *phor1* (Amador *et al.*, 2001), *stgan* (Bachem *et al.*, 2001), *CONSTANS-like* (Martinez-Garcia *et al.*, 2002), *poth1* (Rosin *et al.*, 2003), and s*tbel5* (Chen *et al.*, 2003). In addition, a group of genes has been isolated which exhibited differential expression during tuberization. These were implicated in playing a regulatory role in tuber development although their direct involvement remains ambiguous (Kang and Hannapel, 1996; Kolomiets *et al.*, 2001; Raices *et al.*, 2003). A more high-throughput approach, implementing cDNA-AFLP, provided a large set of differentially expressed transcripts related to early tuber development (Bachem *et al.*, 2000). Most genes mentioned above have been independently linked to tuber development. However, in terms of process control, a coherent sequence of events has not been established thus far. The visible process of potato tuberization involves the transition of a longitudinal growing stolon into an active sink tuber through cell expansion followed by longitudinal cell division of the sub-apical part of the stolon (Xu *et al.*, 1998b). Further tuber growth is primarily due to random cell division and expansion in the perimedullary region (Peterson *et al.*, 1985).

As opposed to tuber development, the starch biosynthesis route in potato has been extensively studied. Sugars are thought to act as the driving force behind the formation and growth of the sink tuber as sucrose is the main photo-assimilate transported from the leaves towards the expanding sink organ (Zrenner *et al.*, 1995). During the rapid growth phase, the active sink tuber accumulates large amounts of storage compounds mainly in the form of starch (Prat *et al.*, 1990). Implementing reverse genetic approaches, most key regulatory genes have been identified although the regulation of starch synthesis has proven to be far more complex than regulation at the transcriptional level alone (reviewed in Geigenberger, 2003; Geigenberger *et al.*, 2004). Starch composition is one of the economically most important characteristics of potato tubers, although other tuber parameters can also be important depending on their targeted industrial application (Ellis *et al.*, 1998). Tuber shape, dormancy, cooking quality and nutritional value are important potato tuber characteristics for which the genetic regulatory mechanisms are still largely unknown. The growing interest in secondary metabolite content in plants, with respect to nutritional value and health promoting properties, demands a more detailed analysis of their function and biosynthesis in order to be able to alter effectively the metabolic content in the potato tuber (Verpoorte and Memelink, 2002).

The active process of potato tuber initiation, tuber growth and tuber filling before a state of dormancy is reached has been extensively described from a physiological point of view. However, these processes require the regulation of large sets of genes from a multitude of different metabolic and signalling pathways. Despite the presence of a set of genes which have been independently shown to be important in potato tuber development, large scale expression analysis of genes linked to metabolic or signalling pathways, thus providing a more complete overview has been lacking. cDNA-microarray technology offers the possibility to study the expression level of such a large set of genes at consecutive time points, thereby potentially providing novel insights into gene regulation and helping to identify genes involved in the regulation of changes in metabolic pathway fluxes (Schena, 1996; Buckhout and Thimm, 2003). Sequencing initiatives using potato cDNA libraries has

yielded a large expressed sequence tag (EST) database (Crookshanks *et al.*, 2001; Ronning *et al.*, 2003) which, in combination with cDNA-microarray technology, provide the possibility to study gene expression in potato on a large scale.

In this paper we describe the construction and use of a dedicated potato cDNA-microarray containing genes likely to play a role in certain aspects of potato tuber development. Processes and metabolic pathways represented on this tuber life cycle array (TLC-array) were studied in relation to early tuber development, to allow a more focused analysis of the genes involved. We present and discuss gene expression data from eight tuber developmental stages in relation to common expression profiles and metabolic pathways with a focus on starch metabolism. Furthermore, the gene expression profiles of newly identified and potentially important developmentally regulated genes are described.

Results and Discussion

Design of a dedicated cDNA-microarray specific for studying the potato tuber life cycle

Our previous work on potato tuberization, using RNA-fingerprinting, has provided a set of differentially expressed transcripts associated with this process (Bachem *et al.*, 2000). For a more detailed analysis of gene expression, a dedicated potato tuber cDNA-microarray was specifically designed to investigate gene expression levels at different stages of the potato tuber life cycle (TLC-array). Besides the fragments isolated using cDNA-AFLP (297 TDFs), the number of arrayed sequences was significantly extended to include genes involved in metabolic and regulatory pathways relevant to potato tuberization, tuber composition and other tuber quality traits. Our primary focus was to analyze genes involved in the metabolism of carbohydrates, amino acids, cell wall, hormones, vesicle trafficking, synthesis of secondary metabolites, and genes related to plant developmental processes. In order to identify the relevant clones involved in these processes a novel automated text mining approach was used. As a starting point, a list of keywords was assembled including names of genes, enzymes and other proteins proposed to be involved in the chosen aspects of tuber development and tuber quality traits based on previous work and a in depth literature review. A large set of non-redundant potato sequences were compared to the GenBank database (Altschul *et al.*, 1990). Blast results were stored and used in an automated screening process for the presence of the selected keywords (Experimental procedures). In this text-mining approach, clones were identified and selected on the basis of high sequence homology to genes that were present in the extensive list of keywords. A total of 1896 cDNA clones were finally selected for arraying (Table I).

To facilitate the interpretation of the results, selected ESTs were assigned to one of the following functional groups on the basis of their biological relevance to potato tuber development: carbohydrate, amino acid, secondary metabolites, hormone metabolism, plant development and transcriptional regulation, cell structure, signalling, transport or TDFs with no significant homology to known genes. These general categories include genes involved in many independent metabolic pathways on the basis of the KEGG Pathway database

(http://www.genome.jp/kegg/pathway.html). ESTs that could not be unambiguously assigned to any of these categories were classified as undefined. Table I provides an overview of the origin and the number of selected clones spotted on the TLC-array subdivided into the functional categories. Although the number of available ESTs has grown substantially since the early database release (august 2001), and hypotheses on mechanisms controlling tuber development and metabolic fluxes are continually changing, we consider that the chosen genes remain a relevant and representative set, well suited to study tuber development and associated tuber quality characteristics at the transcriptional level.

Table I: Design of the potato tuber life cycle cDNA-array (TLC-array) with clone origin and functional classification

CATEGORY	***CLONE ORIGIN***								
	cStA[a]	cStB[b]	cStC[c]	cStD[d]	cStE[e]	cStS[f]	TDF[g]	Gene clone[h]	Total. Category
	No.clones	No.clones	No.clones	No.clones	No.clones	No.clones	No.clones	No.clones	No.clones
Carbohydrate Metabolism	89	62	4	12	50	54	21	14	***306***
Amino Acid Metabolism	115	67	4	14	37	56	5	0	***298***
Secondary metabolism	57	105	7	12	13	76	11	1	***282***
Structural	29	27	1	1	11	28	11	12	***120***
Transport	23	14	1	4	17	28	8	1	***96***
Signal Transduction	38	25	3	9	12	52	17	1	***157***
Transcriptional Regulation & Development	24	16	0	7	12	28	17	0	***104***
Hormone Related	29	22	3	8	19	39	6	0	***126***
TDFs with no function homology	0	0	0	0	0	0	148	0	***148***
Undefined	53	64	1	21	10	49	53	8	***259***
Total Clone Origin	457	402	24	88	181	410	297	37	***1896***

TIGR cDNA libraries from different potato organs; [a]stolon,[b]leaf, [c]sprouting eyes, [d]dormant tuber, [e]microtuber, [f]sprouting (shoots), [g]differentially expressed cDNA-AFLP fragments during tuber development (Bachem *et al.*, 2000),[h]cloned and annotated potato genes from a wide variety of sources.

Differential gene expression profiles during tuber development

The availability of a synchronous potato tuber induction system is vital when investigating differential gene expression in a developmental time series. In the system described here, plant material was obtained from *S. tuberosum* L. cv. Bintje, grown in a controlled environment under long day conditions and subsequently switched to short day conditions to promote synchronous tuberization. RNA targets were isolated from eight developmental time points, ranging from non-induced stolons, induced stolons, swelling stolon tips, tuber initiation stage and four different tuber growth stages (Figure 1). Recreating

the natural conditions for tuber development allowed the harvest of specific tuber developmental stages from several plants simultaneously, providing a synchronous *in vivo* developmental series.

Previously, an *in vitro*-based system was used in which final tuber diameter did not exceed 1 cm and tuber induction was strongly dependent on the presence of high sucrose levels in the medium (Hendriks *et al.*, 1991). Nevertheless, in a comparison between the data presented here and gene expression analyzed previously using the *in vitro* system (Bachem *et al.*, 2000), we found similar expression profiles for genes analyzed in both systems (data not shown). This confirms the synchronous nature of the *in vivo* system with the added value of secondary tuber growth and whole plant dynamics.

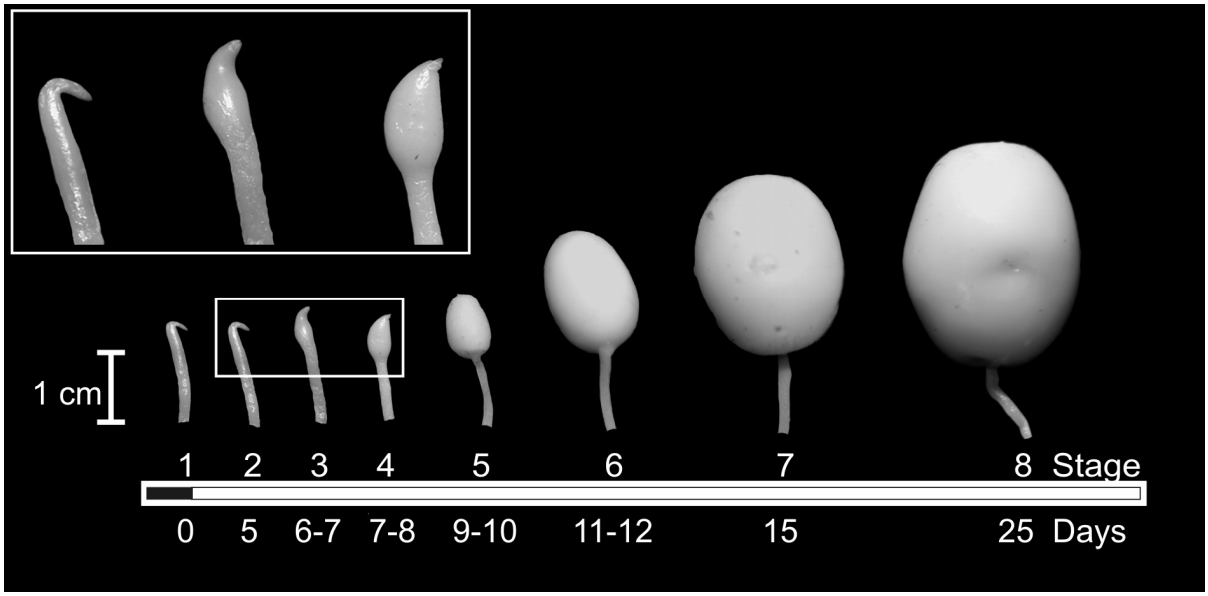

Figure 1; Potato tuber developmental stages (1-8) harvested in a time period of 25 days after a switch from 16h light (black box) to an 8h light period (white box). Top left corner enlargement of stages 2, 3 and 4. Stage 1; Stolon tip of potato plant grown under long day conditions (16h). Stage 2; Stolon tip harvested 5 days after the switch to short day (5 DAS) conditions (8h). Stage 3; First visible swelling harvested 6-7 DAS. Stage 4; Tuber initiation (7-8 DAS). Stage 5; Tuber size 1 with diameter of ~ 0.8 cm (9-10 DAS). Stage 6; Tuber size 2 (11-12 DAS). Stage 7; Tuber size 3 (15 DAS) and Stage 8; Tuber size 4 (25 DAS).

To determine gene expression levels during development, the eight developmental time points were hybridized individually on the TLC-array against a common reference sample (dataset A). All hybridizations were repeated in a swap-dye experiment. Data were collected, normalized and validated as described in Experimental procedures. The resulting dataset ('Supplementary material' Table S1) was compared to a biological repeat (dataset B) ('Supplementary material' Table S2). Pearson's correlation of the identical harvest points between both datasets were in general high (stage 2, 0.9; stage 3, 087; stage 4, 0.76; stage 5, 0.35; stage 6, 0.61; stage 8; 0.6), the only exception being stage 5 that had a correlation coefficient of only 0.35 over the biological repeats. Repeat B of stage 5 is more similar to both stage 6 profiles (A; 0.6 and B; 0.49 respectively), indicating that the harvested tubers in stage 5 of the one repeat was further in its developmental program than expected. As a whole, expression ratios from both experiments were considered confirmatory particularly with

respect to the observed trend of gene expression. Thus, further analysis was performed on the larger dataset A that consists of the eight developmental time points allowing the detection of more subtle changes. Relative expression levels of 1315 genes of the validated set ('Supplementary material' Table S1) are presented as a matrix based cluster diagram (Figure 2A).

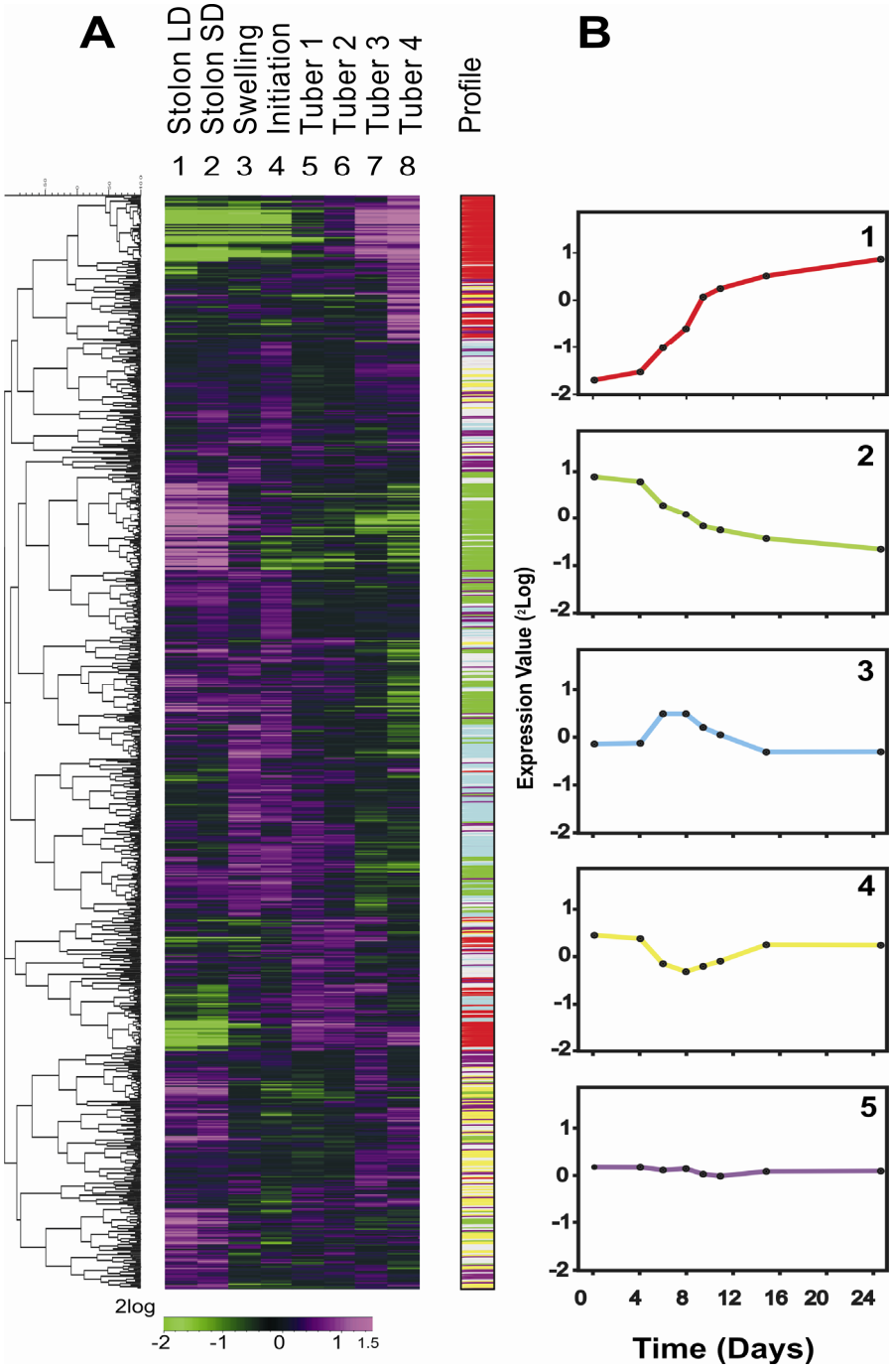

Figure 2; Relative gene expression data of 1315 ESTs of the 8 potato tuber development stages (Figure 1) clustered and grouped according to their relative expression profile (A-B). (A) Hierarchical clustering of developmental stages (1-8) with on the right a colour bar, grouping all genes in 6 major expression profiles; ■ Up-regulated, ■ Down-regulated, ■ Transient up-regulated, ■ Transient down-regulated, ■ Constitutive expression and ■ Rest group. (B) Graphs (1-5); Averaged expression patterns of the identified expression profile groups during tuber developmental stages (1-8).

Genes following a similar expression pattern during the time points under investigation are clustered together. Major expression profiles were identified based on self-organizing maps and genes were empirically assigned into five major colour-coded profile groups and a rest group (vertical colour bar, Figure 2A). Expression profile identification was focused on the early stages of tuber development since expression levels in the final tuber stage show in general a down-regulated trend except for a small set of storage and other high accumulating proteins. The five main groups include up-regulated (red; 211 members), down-regulated (green; 263 members), transiently up-regulated (blue; 201 members), transiently down-regulated (yellow; 150 members) and constitutively expressed genes (purple; 202 members). Genes which did not display one of the above expression profiles were assigned to a rest-group (grey; 288 members). Genes in this group show either a distinct expression profile found in only a limited number of genes or demonstrated an irregular or noisy expression pattern during development. On the basis of these patterns, generalized conclusions cannot be drawn about their function and they can therefore only be investigated further on a case-by-case basis.

During the first two time points (Figure 1; stage 1 and 2), between which the plants were transferred from long-day to short-day conditions, only a small fraction (4%) of the genes expressed in stolons change their expression level significantly (≥ 2-fold). After the switch to short day conditions, stolons showed synchronous swelling after 5 days (Figure 1; stage 3). The transition between stage 2 and 3 is more dramatic and is accompanied by a ≥ 2-fold change in expression levels in 15% of the genes represented on the array. Expression levels of genes at stolon swelling (Figure 1; stage 3) generally continue their up- or downward trend in stage 4, which represents tuber initiation (Figure 1; stage 4). After the developmental switch of tuber organogenesis (Figure 1; stages 3 and 4), gene expression levels in the newly formed tuber and subsequent growth stages (Figure 1; stages 5 to 8) either return to their basal level or continue the down- or up-regulated expression pattern. In Figure 2B (graphs 1-5), averaged expression profiles have been calculated from the assigned profile groups. Surprisingly, down-regulation of gene expression during the studied developmental time points is the most common expression profile. Nevertheless, the up-regulated pattern has, on average, a much higher margin of change in relative expression levels as exemplified by comparing averaged ^{2}log expression ratios (Figure 2B; graphs 1 and 2). Both transient increases and decreases (Figure 2A; graphs 3 and 4, respectively) follow more subtle changes in expression level in comparison to the up-regulated profiles but they could nevertheless be identified as discrete patterns. The transient change in gene expression occurs most frequently during stolon swelling and tuber initiation. This developmental phase is characterized by changes in the plane of cell division and expansion resulting in longitudinal swelling of the subapical region (Xu *et al.*, 1998b). The large number of genes that share a common expression profile indicates a high level of coordinate transcriptional control of metabolic or developmental pathways during tuber initiation and tuber growth.

Regulation of gene expression related to development and metabolic pathway fluxes

As described earlier, each EST on the array had been assigned to a functional category based on its sequence similarity (Table I). Analysis of the distribution of the different functional classes over the five expression profiles during tuber development provides an insight into possible changes in metabolic fluxes and the coordinate transcriptional regulation of developmental processes. Three out of the ten functional categories show a clear preference for a common expression profile identified during tuber development. Firstly, the carbohydrate group, which consists for a large part out of genes dedicated to the synthesis of starch, has many genes that show an up-regulated trend (28%). The up-regulated profile of certain starch synthesis genes was characterized by a strong increase during the initial stages of tuber formation after which a relatively high expression level is maintained until final tuber size is reached. A logical explanation for this observation is an increased rate of starch synthesis at tuber induction and subsequent tuber growth. The switch from apoplastic towards symplastic sucrose unloading at tuber organogenesis results in increased cellular sucrose availability during tuber development (Viola *et al.*, 2001). As a result, carbohydrate metabolism and sucrose-linked pathways have to adjust their flux accordingly and are dedicated towards the synthesis of starch.

Another functional category for which a large subset of genes show coordinated expression changes during development contains the structural genes (e.g. cell wall, cytoskeleton). More than half of these genes are down-regulated either during the whole developmental program or temporarily during tuber initiation (47% and 21% respectively). Down-regulation of structural genes may indicate an altered requirement for cell wall synthesizing enzymes and proteins during the transition between stolon growth and stolon swelling. It is important to note that the observed down regulated expression profile may in part, be due to a dilution of transcripts during the later stages of tuber development. Genes solely expressed in meristematic tissue for example, or in cell-types other than parenchyma cells, can appear to be down-regulated since the ratio of these cell types decrease with the growth of the tuber. However, such effects are likely to become only significant at the later stages of tuber development.

The third functional category that has a more than average proportion of genes following a specific expression profile is the amino acid group. In our results, a relatively large number of amino acid synthesis genes are down-regulated in comparison to the number of up-regulated genes (22% and 10%, respectively). However, an even larger proportion of amino acid related genes (37%) show a transient increase during tuber initiation stages. A possible explanation for the observed temporal increase in transcript levels found during tuber organogenesis may be an elevated requirement for free amino acid levels to maintain cellular osmoticum (Fernie *et al.*, 2002a). Roesnner-Tunali *et al*., (2003) linked sucrose levels to the regulation of *de novo* amino acid synthesis at the transcriptional level. Analysis of plants with restricted sucrose supply showed an inverse relationship between the levels of sucrose and free amino acids.

Over-representation of a common expression profile in a specific metabolic route during tuber development may indicate at least partial regulation of such a pathway through transcriptional control. However, general conclusions on changing metabolic fluxes cannot be drawn without additional metabolite data or enzymatic activity assays.

Detailed analysis of gene expression levels in the starch biosynthesis pathway

Implementing a dedicated array approach as presented in this paper, provides the possibility to study expression levels of many genes within the same metabolic pathway and can therefore provide additional information on metabolic flux or key transcriptionally regulated steps. A simplified schematic pathway of the key-enzymes involved in starch biosynthesis and their relative expression level is presented in Figure 3 ('Supplementary material' Table S3). Different gene family members, isoforms or subunits are identified, where possible. The levels of transcription of genes at each of the eight developmental stages are represented as a colour bar next to the enzymatic steps. The ^{2}log-based colour scheme was optimized to allow differentiation of subtle expression differences. Many genes directly involved in the sucrose to starch synthesis pathway are strongly up-regulated from the swelling stolon stage onwards, although increased expression can already be observed prior to stolon swelling. During tuber organogenesis (stage 3 and 4), higher levels of sucrose import coincide with increased starch synthesis as exemplified by increased transcript levels of several genes involved in sucrose-starch metabolism (Figure 3; reaction 2, 6, 10, 11, 12 and 13). The genes coding for enzymes directly associated with sucrose cleavage (Figure 3; reactions 1 and 2) show changes in expression during the early stages of tuber development that correlates with a switch in the predominant sucrose breakdown route and metabolic flux directed towards increased starch synthesis (Appeldoorn *et al.*, 1997). Sucrose synthase (SUSY) transcript levels (reaction 2) increases dramatically (35-fold increase at tuber initiation, stage 3) reaching a peak during stages 5 and 6 (60-fold increase compared to stage 1). The elevated level of SUSY gene expression coincides with a down-regulation and a transient decrease of transcript levels of two soluble acid invertase isoenzymes (reaction 1), whilst the cell wall and neutral invertase remains relatively constant. Removal of sucrose cleavage products is important to maintain a high rate of starch synthesis. This is in agreement with our finding of a strong correlation in expression levels between fructokinase II (FK; reaction 6) and SUSY (reaction 2). This reaction is a good example of differential expression of gene isoforms in which three FK isoforms are represented by four different clones. Two clones (BI176914, Z12823) correspond to the potato FK II gene (Smith *et al.*, 1993), whilst the other two ESTs (BF187854, BG593589) have homology to two different tomato *(Solanum Lycopersicum*) FK isoforms: FK I (Kanayama *et al.*, 1997) and FK III (German *et al.*, 2003) respectively. Both FK II clones on the TLC-array have identical expression profiles and are strongly up-regulated. The expression profiles of FK I and III homologues, appear to be negatively correlated. FK I shows higher levels of expression during the later stages of tuber growth while the FK III homolog has an early peak of expression at tuber initiation after which expression levels decrease.

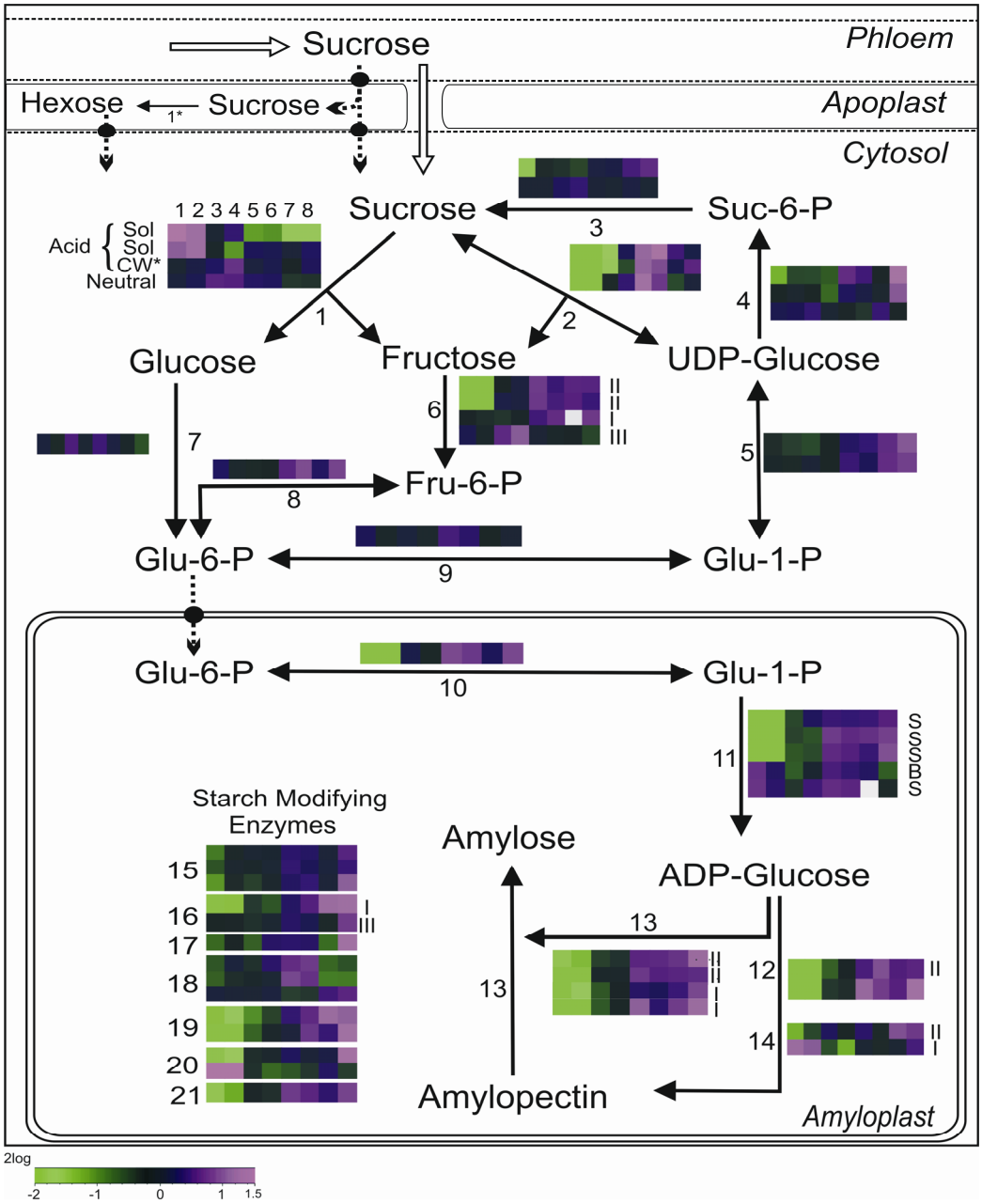

Figure 3; Schematic drawing of part of the starch synthesis pathway with relative expression levels of genes represented as false colour boxes during the tuber development stages 1-8 (Figure 1). Black arrows indicate enzymatic reactions. Dashed arrows represents active transport of metabolites across cellular membranes and open arrows represents symplastic transport. Enzymes or genes involved in enzymatic reactions are numbered (1-21) and gene isoforms or different subunits are indicated where possible. Genbank accession numbers provided for each reaction correspond to the gene profiles in the figure from top to bottom. Reactions; 1 = invertase (BG597287; AW906173; BG593864; BG600876); 2 = sucrose synthase (BI178251; CO654077; BI178415) ; 3 = sucrose-P phosphorylase (BE471979; BE343008); 4 = sucrose-P synthase (X73477; BG593948; BG886873); 5 = UGPase (D00667; BI178018); 6 = fructokinase (I,II and III) (Z12823; BI176914; BF187854; BG593589); 7 = hexokinase (BF053338); 8 = phosphoglucose isomerase (BI179391); 9 = cytosolic phosphoglucomutase (BF052510); 10= plastidic phosphoglucomutase (BI176542); 11 = AGPase (B,S) (AW906205; CO654075; CO654076; X55155; X61186); 12 = soluble starch synthase (BI176976; BI177076); 13 = granule bound starch synthase (I, II) (X87988; BI177463; BI178087; BF186932); 14 = starch branching enzyme (I,II) (AJ000004; BI176741); 15 = starch associated R1 (BF187950; BG595386; BG593226) 16 = isoamylase (I,III) (BI176864; BG595915); 17 = α-amylase (BG595422); 18 = β-amylase (BE472838; BI179340; AW905940); 19 = pullulanase (BI179274; BG597644); 20 = starch phosphorylase (BI177686; AY356214); 21 = disproportionating-enzyme (D-enzyme) (X68664).

Another interesting observation was the strong increase of relative expression levels of plastidic phosphoglucomutase (PGM*)*, and not cytosolic PGM. This finding is a strong indicator for the active import of Glucose-6-P into the amyloplast as described by Tauberger *et al*. (2000). ADP-glucose pyrophosphorylase (AGPase; reaction 11), a key regulatory enzyme of starch synthesis is a hetro-tetrameric enzyme comprising small (B) and large subunits (S). Three ESTs, corresponding to large (S) subunit proteins show strong increases in expression during tuberization. Other ESTs, with homology to another large (S) and a small (B) subunit protein, show a different profile and are expressed in all developmental time points with a small decrease during swelling. ADP-glucose molecules are the building blocks of starch. Soluble starch synthase (SSS; reaction 12), together with the activities of branching enzyme (BE; reaction 14) and granule bound starch synthase (GBSS; reaction 13), synthesize the two main components of starch; amylopectin and amylose. In our experiments we observed that genes coding for SSS and GBSS show a strong increase in expression after the switch to short days and an even stronger increase at tuber organogenesis and further tuber development. Two different forms of potato starch branching enzyme (SBE) are present on the TLC-array. SBE I shows high expression levels in both stolon stages followed by a transient decrease during tuber organogenesis, whilst SBE II shows a strong up-regulation throughout tuber development. The expression profile of SBE II is consistent with the role of the enzyme in starch synthesis where a reduction in transcript levels have been shown to severely alter starch morphology (Schwall *et al.*, 2000). Another example of differential regulation between related genes concerns the isoamylase isoforms (Figure 3; reaction 16). Isoamylase I shows strong up-regulation whilst isoform III has a more irregular pattern. Recently isoamylase isoform I and II have been reported to be associated with starch granule initiation (Bustos *et al.*, 2004). A definite role for isoamylase III in starch metabolism has not yet been assigned. In both cases these results indicate an independent transcriptional regulation of gene family members or isoforms in potato tuber development. Differentiation between expression profiles of gene family members involved in primary or secondary metabolic pathways may prove useful to predict the key regulatory genes. However, cross-hybridization of closely related genes with high levels of sequence homology should always be taken into account as an interfering factor when using cDNA-microarrays. Especially transient down-regulated profiles can be the net result of two counteracting expression profiles, down-regulated and up-regulated. In these instances, two genes may have similar functions but are expressed in different tissues in either stolon or tuber. Although individual gene expression profiles of many starch synthesis genes have been separately published (reviewed in Kossmann and Lloyd, 2000; Fernie *et al.*, 2002b; Geigenberger, 2003), the analysis presented in this paper gives a unique overview of transcriptional regulation of starch synthesis during tuber development.

Analysis of tissue specificity for genes on the TLC-array

The genes that show differential expression during tuber development may be highly process specific and therefore expressed in the stolon or tuber. Data on tissue specificity of

the genes analyzed during tuber development provides additional information that can be helpful in understanding gene function. RNA was isolated from a representative set of potato tissues (leaf, stem, developing flower bud, root, stolon bearing a tuber, non-tuberising stolon, tuber, dormant tuber and berry) and hybridized to the TLC-array. A common reference sample consisting of a mixture of tissues was used in all experiments. Gene expression data ('Supplementary material' Table S4) of the tissues were analyzed and scored for tissue specificity as described in Experimental procedures. Based on the level of tissue specificity of the expression patterns, genes were classified in eight categories ranging from specific expression in a single organ to similar expression levels in all of the eight tissues investigated. The largest group (699 genes) consists of genes that do not show tissue-specific expression in the tissues investigated. Genes with significantly higher expression in only one out of the eight tissues were labelled as uniquely expressed (212 genes). The third largest group shows high expression in two out of the eight tissues analyzed and consisted of 137 genes. The number of expressed genes in the remaining classes of tissue specificity steadily decreases. In the last group, containing genes with high expression in seven out of the eight tissues, only 12 genes were found. Genes that were classified as either uniquely expressed or expressed in two out of the eight tissues were analyzed further (Table II).

Table II: Tissue specificity of genes on the TLC-array with either unique or relatively high expression in only two potato tissues analyzed

Tissues	**Unique expression and number of genes highly expressed in two out of the eight tissues**							
	Tuber[1]	Berry	Stolon	NTS[2]	Root	Stem	Leaf	DF[3]
Tuber	51							
Berry	23	16						
Stolon	15	2	28					
NTS	14	1	2	22				
Root	3	5	4	2	6			
Stem	2	1	0	1	2	9		
Leaf	6	4	2	1	0	8	47	
DF	13	1	2	13	0	1	9	33

[1] Tuber + Dormant tuber; [2] Non-tuberizing stolon tip; [3] Developing Flower bud (DF).

Using the TLC-array, unique expression in tubers is found for 51 genes, while in root and in stem only 6 and 9 genes respectively are uniquely expressed. Interestingly, tuber and berry, both major sink organs, share 23 genes that are highly expressed in these two tissues only and have functions generally associated with storage and defence ('Supplementary material' Table S4). Expression of a number of genes in both non-induced stolons and developing flower buds (13 genes) suggests involvement in developmental programs of young organs. Furthermore, these two tissues have a relatively high number of genes uniquely expressed in only one tissue (non tuberising stolon, 22 genes and developing flower bud, 33 genes) indicating a specific role in their respective tissue or developmental stage. Genes showing strong changes in relative expression levels during tuber development (up-

and down-regulation), appear to have a higher degree of tissue specificity compared to other expression profiles under investigation ('Supplementary material' Tables S1 and S4).

Gene expression during development in relation to tissue specificity

A large proportion of genes (47%) on the TLC-array show differential patterns of gene expression in the tissues studied. The expression data obtained, from both the tissue specificity experiment and the tuber developmental series, led to a new approach to explore the possible association between tissue specificity and gene expression during tuber development. Principal component analysis of expression data, obtained from the potato developmental series, results in a plot with the eight harvest time points in consecutive order (Figure 4A). The first two components accounted for > 82% of all observed variance during tuber development. In the plot, changes in gene expression follow a clear trajectory from initial stolon stages (stages 1 and 2) to the formation of a mature tuber (stages 7 and 8). Figure 4B shows the corresponding distribution of genes from the same PCA of the developmental series. The genes have been colour-coded according to their relative expression profile as shown in Figure 2A. As expected genes that are strongly up-regulated during development (red) are primarily found on the right hand side of the plot (PC 1) associated with the later stages of tuber development (Figure 4A). Down-regulated genes (green) are distributed on the far left hand side of the plot in accordance with their relatively high expression during stages 1 and 2. The two groups with transient expression profiles (blue; transiently up and yellow; transiently down) were, in general, more equally expressed and are more centred with respect to PC1; however, both groups are clearly separated by PC2. Genes that are constitutively expressed (purple) or genes that were assigned to the rest group (grey) are clustered in the centre of the plot.

In order to explore the possible correlation between tissue specificity and developmental expression profiles, data from both experiments were combined in a single plot. For this purpose tissue expression patterns were weighted according to the developmental profiles as distributed in Figure 4B, and were plotted together with the developmental stages in a single representation (Figure 4C). In this way, an overall association between gene expression during tuber development and expression in potato tissues can be established. When no correlation exists between temporal and spatial expression patterns, the different tissues would be positioned near the centre of the plot. However, leaf, stem, root and developing flower bud were positioned on the left hand side of the plot in proximity to the non-tuberising stolon and swelling stages (Figure 1; stages 1 to 3). Thus, these tissues share more than an average proportion of genes that are preferentially expressed during the early stages of tuber development. In contrast, tuber and dormant tuber expression patterns are more associated with the later stages of tuber development as shown by their position on the right hand side of the plot. Expression patterns of stolon and berry are more neutral with respect to developmental gene expression, although berry is closer to tuber than to stolon stages in this analysis.

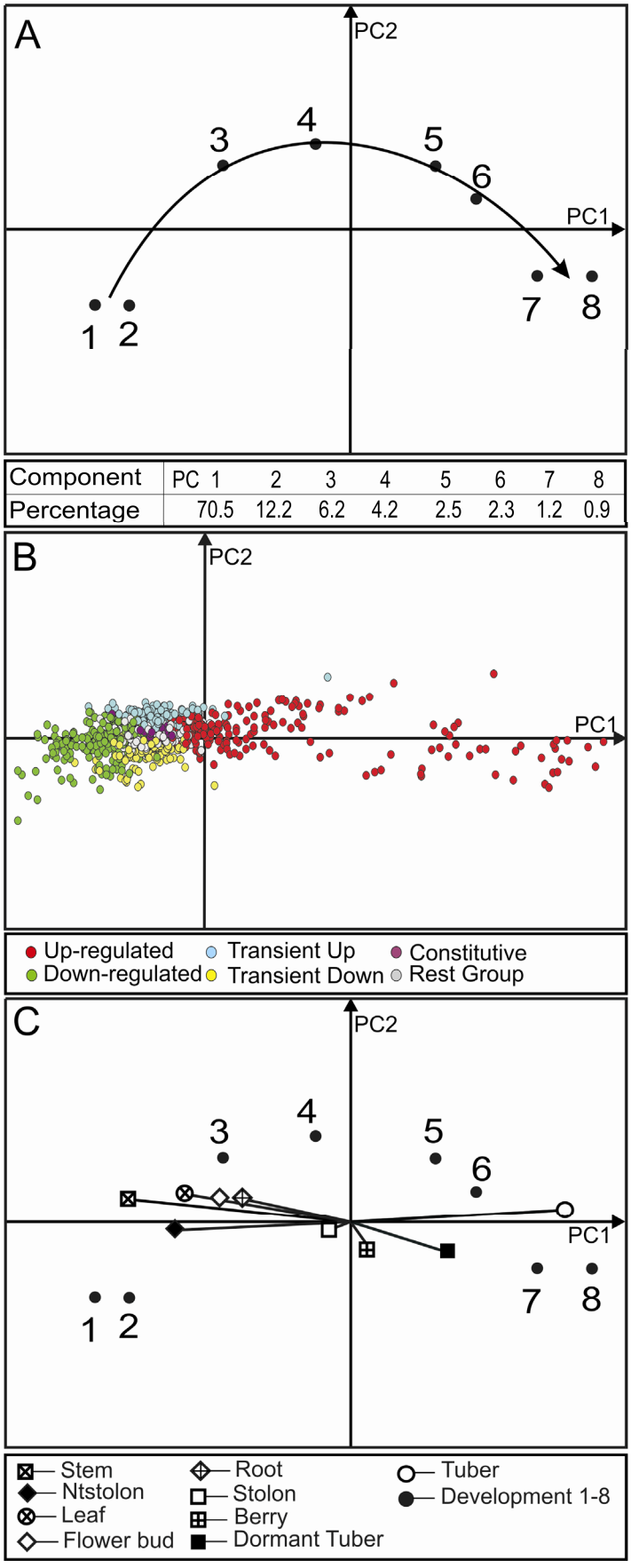

Figure 4; Principal component analysis (PCA) of potato tuber developmental stages (1-8) linked to potato tissue expression patterns (A-C). (A) PCA plot of the eight potato tuber developmental time points. The drawn arrow indicating the developmental trajectory. (B) Corresponding plot of expression profiles of 1315 genes from the eight potato tuber developmental time points. Individual genes are colour-coded according to their assigned expression profile (see Figure 2A). (C) Tissue expression patterns were weighted according to their tuber developmental profiles as distributed in Figure 4B, and plotted together with the developmental stages, visualizing a possible linkage between gene expression during tuber development and in potato tissues.

From expression data to candidate gene

Based on the differential expression profiles identified during tuber development, the degree of tissue specificity and the sequence homology to annotated sequences, genes can be marked as potentially important in the process of potato tuber formation. Depending on the researchers' interest, candidate genes can be further analyzed to elucidate their role during tuber development or associate their functions with processes that determine tuber quality traits. Here, examples of candidate genes are described that are linked to early tuber developmental processes and also represent the four major differential expression profiles identified. Expression profiles of these candidate genes were confirmed using quantitative RT-PCR (Figure 5 A-D).

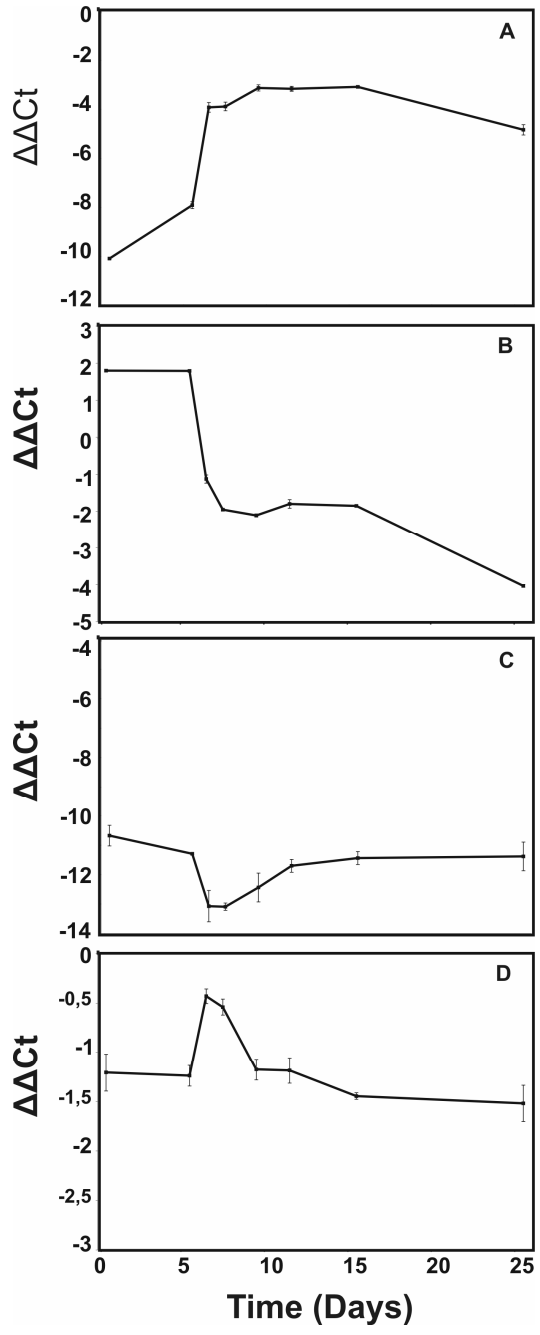

Figure 5; Quantitative RT-PCR analysis of RNA extracted from eight tuber developmental stages harvested in a time period of 25 days after a switch from 16h to 8h light period (DAS). ΔΔCt values were calculated using ubiquitin expression levels as a control. Error bars show the standard error of the mean of three measurements. Expression profiles of genes (A) gibberellin 2-oxidase homolog; BI176613, (B) *adr11-2* homolog; BE339784. (C) gibberellin 2-oxidase homolog; BG096003 and (D) *arcA* homolog; BI177453.

One of the few early up-regulated genes, prior to visible swelling, showed high homology to a GA2-oxidase (Figure 5A; BI176613), which is involved in GA breakdown (Thomas *et al.*, 1999). The strong early increase in transcription levels of the gene coding for a GA2-oxidase may reflect the mechanism by which the required lowering of active GA levels in the stolon tip is accomplished prior to tuberization. Interestingly, we found an additional GA2-oxidase homolog (BG096003) that was transiently down-regulated at tuber organogenesis (Figure 5C). Based on its sequence homology this GA2-oxidase appears to belong to a separate class of GA 2-oxidases that hydroxylate C_{20}-GA precursors and not C_{19}-GAs (Schomburg *et al.*, 2003).

The down-regulated profile shows a significant decrease of transcription levels at tuber organogenesis and one of the strongest examples of such an expression profile is a gene with homology to *adr11-2* (Figure 5B; BE339784). *adr11-2* (auxin down-regulated) was first identified in soybean and transcript levels were reduced up to 15 fold in seedlings after treatment with auxin (Datta *et al.*, 1993). To our knowledge, no clear function for this gene has been established thus far. In our experiment, the *adr11-2* homologue was found to be expressed primarily in non-tuberising stolons and developing flower buds; both young developing organs ('Supplementary material' Table S4). Koda and Okazawa, (1983) found high levels of auxin in the swelling stolon tip after which auxin levels decreased. This is in accordance with the observed decrease in expression levels of the *adr11-2* homologue during stolon swelling (stage 3). Auxins (IAA) are known to have an effect on tuber development. Xu *et al.*, (1998b) observed earlier tuberization when IAA was applied to single node cuttings in tuber-inducing medium. The importance of auxin dependent transcriptional regulation during tuber development is further underlined by the finding of numerous auxin responsive genes in the different expression profiles ('Supplementary material' Table S1).

Another candidate gene (BI177453) that appears to be regulated to some degree by auxin has homology to the *arcA*, a member of the auxin regulated gene family containing a GTP-binding site. Expression of *arcA* has been shown to be induced after auxin treatment in tobacco BY-2 cell lines and was given a putative role in the induction of cell division (Ishida *et al.*, 1993). In our experiment, the EST with homology to *arcA* shows a transient increase during tuber organogenesis (Figure 5D). During this phase increased cell division takes place within the pith (Xu *et al.*, 1998b). This gene appears to be a good candidate for involvement in regulating the rate of cell division at tuber organogenesis. The above described candidate genes are representatives of the four major differential expression profiles found within this experiment. These genes and other putative candidate genes available within the dataset are likely to prove important with respect to potato tuber development. However, the determination of their precise function requires further research. Not only does the expression data on potato tuber development help in the further understanding of the biological mechanisms involved, the identification of key regulatory sequences may also facilitate the targeted alteration of metabolic routes in potato tubers for industrial purposes and will provide markers for precision selection in breeding programs.

Experimental procedures

Clone selection and microarray preparation

In order to implement an automated screening process for the presence of keywords in the blast results, a PERL script was written. The complete set of unique sequences that was used derived from TIGR database release August 2001 (Crookshanks *et al.*, 2001; Ronning *et al.*, 2003). This sequence set was comprised of 9996 ESTs and 6346 tentative consensuses (TCs). Sequences were compared (BLASTX) to the non-redundant GenBank database (Altschul *et al.*, 1990). For each sequence, the first 10 blast hits were stored and automatically searched for the presence of keywords. Blast results containing one or more keywords were flagged and manually reviewed in order to filter out false hits and to assess the quality of the alignments and homology levels. For each unique sequence selected, a representative EST was selected for arraying. Flagged sequences that showed low homology to desired genes, related to the potato tuber life cycle, were still selected but were categorized as undefined. The same category also contained genes with known biological functions but that could not be assigned to any of the other functional categories. The selection list based on the keyword approach was extended with 297 cDNA-AFLP fragments (TDFs) deriving from potato *in vitro* tuberization experiments (Bachem *et al.*, 2000) and 37 genes deriving from other sources. cDNA fragments selected for arraying were obtained by PCR amplification on isolated plasmid DNA. Plasmid DNA was isolated using a robotic sample processor (Genesis 150, Tecan) in combination with the Qiaprep® plasmid miniprep kit. cDNA clones derived from TIGR constructed cDNA libraries were amplified by polymerase chain reaction (PCR) using 5' 6 bp extended universal T3 and T7 primers (Isogen). pGEM-T cloned cDNA-AFLP fragments (TDFs) were amplified using M13 forward and reverse universal primers (Eurogentec). The PCR program used 30 cycles of 30 sec at 94°C, 30 sec at 55°C, followed by 2.5 min at 72°C for TIGR derived clones and 1 min at 55°C for TDFs. PCR products were checked for single band amplification before purification using the QIAquick™ Multiwell PCR purification kit (Qiagen) and eluted in 100 µl of 0.1xTE, pH 8.0. In addition, PCR products of three negative control clones (each in 8-fold) were added and consisted of non-plant genes used for background evaluation; yeast aspartate kinase (J03526), imidazole- glycerol-phosphate dehydratase (Z75110) and phosphoribosylamino-imidazole carboxylase (Z75036). As a positive control, for normalization purposes, the complete coding sequence of the firefly luciferase gene (M15077) was included (22-fold). Furthermore, three partial luciferase clones (5', middle and 3', each 8-fold respectively) were included to monitor labelled cDNA integrity. Samples were dried to completion, resuspended in 10 µl of 5 x SSC and transferred to a 384-format plate for spotting. Spotting was done with the PixSys 7500 (Cartesian Technologies) arraying robot on GAPS aminosilane coated microscope glass slides (Corning) using a 3 quill-pin print-head. Spotting volumes were about 0.5 nl resulting in a spot diameter of 120µm at a pitch of 160 µm. After arraying, slides were processed as described by van Doorn *et al.,* (2003).

Plant Material

For the potato tuber developmental series (Figure 1), approximately 100 *in vitro* potato plants (*Solanum tuberosum* L. cv. Bintje) were maintained on Murashige and Skoog medium (Murashige and Skoog, 1962) for two weeks under controlled conditions with a 16 h light period at 22ºC. *In vitro* plants were transferred to soil and maintained in a climate chamber for a period of six weeks (16 h light at 20ºC and 8 h dark period at 18ºC). The non-tuberising stolon tips were then harvested 3-4 hours into the light period. Remaining plants were transferred to short day conditions (8 h light and 16 h dark period). Stolon tips and developing tuber stages (2 to 8) were harvested during a period of 25 days after the switch to short day conditions (DAS). All stages were harvested 3-4 hours into the light period. For the potato tissue experiment, all tissues were collected from *S. tuberosum* L. cv. Bintje plants except berry tissue, which was harvested from several genotypes of the progeny from a potato crossing population (CxE) (van Eck *et al.*, 1995). Each tissue was harvested from at least 6 individual plants. Non-tuberising stolon tips were harvested from approx. 6 week old greenhouse grown plants. Fully expanded leaf, stem, stolon, root and tuber (> 3 cm diameter) were harvested at the same time from 12 week old greenhouse-grown plants. Developing flower buds and berries were harvested from tuber bearing field grown plants. Dormant tubers derived from a field harvest were stored for four weeks at 4ºC.

Sample preparation and labelling

Total RNA was isolated as previously described by Bachem *et al.,* (1996). Poly(A)$^+$ mRNA was obtained in two rounds of purification on oligo(dT) columns (Amersham Pharmacia). Poly(A)$^+$ was spiked with luciferase mRNA (Promega) and reverse-transcribed as described by van Doorn *et al.,* (2003). After RNA degradation, cDNA samples were resuspended in 10µl Sodium Carbonate buffer (pH 9.3) and appropriate amounts were either labelled with Cyanine 3 (Cy3) or Cyanine 5 (Cy5) fluorescent dyes (Amersham Pharmacia). Samples were washed in two rounds of ethanol precipitation and finally dissolved in 5µl of de-ionized H_2O. For the developmental range series, a single reference sample (consisting of a mixture of harvest time points 2-6 and 8) was used for each series of hybridizations, including swap dye experiments, in order to allow direct comparison of expression ratios between the different developmental stages. In the biological repeat (set B) a reference was made from the same time points as the original. Hybridizations for time points 1 and 7 were not performed. The reference sample used in all potato tissue hybridizations (including swap dye) was a mixture of the following tissues; leaf, tuber, developing flower, stem and root.

Hybridization and Data acquisition

After pre-hybridization for two hours at 42ºC in hybridization buffer (50% formamide, 5x Denhardt's reagent, 5x SSC, 0.2% SDS and 0.1 mg/ml denatured fish-DNA), slides were washed in MQ water and isopropanol and dried by centrifugation (2 min , 470 x

g). During hybridization the array was incubated for 18 hours at 42ºC in a custom-built hybridization chamber. The hybridization mixture (total volume 90 µl) contained (final concentrations); 50% formamide, 5x Denhardt's reagent, 5x SSC, 0.2% SDS, 0.1 mg/ml denatured fish-DNA and both Cy3- and Cy5-labelled samples at a concentration estimated on the basis of 8 ng of the initial mRNA per µl of hybridization mixture. Routinely, all hybridizations were repeated in a swap-dye experiment. After hybridization, slides were washed and scanned on a ScanArray 3000 (Packard Bioscience) as previously described by Van Doorn *et al.,* (2003). Total pixel intensities were obtained for each spot with the AIS software package (Imaging Research).

Microarray data analysis

Raw data was processed using the Microsoft® Excel software package. Median background (BG) values were calculated from the non-plant control clones (J03526, Z75110 and Z75036, each 8-fold) and were subtracted from each duplicate set separately. Channel normalization was done per duplicate set using the mean hybridization signal (spiked mRNA) of the full-length luciferase control clones (M15077, 22-fold). Expression ratios (R(n,t)) were calculated for each clone (n) in each sample (t) when BG subtracted spot intensity was above threshold (0.5xBG). Median ^{2}log ratios were calculated per sample, including swap dye results and duplicates, if no more than two values were missing. In a comparison of the developmental expression profiles between the biological repeats (A(t = 8) and B(t = 6)) a validated set of 1315 clones was extracted, allowing a maximum of two missing values based on dataset A, which was used in all further analysis. Data were exported into the software package GeneMaths™ (Applied Maths) for clustering, SOM and PCA analysis. After column standardization (subtract column average) clustering was done using Pearson's algorithm. PCA analysis was performed on the expression profiles of the developmental series (set A: n = 1315, t = 8) after centring of the samples (subtracting average) (Figure 4A). The corresponding loading matrix (1315 clones) was extracted for the first two components (PC1 and PC2) and combined with the expression patterns of the tissues (t = 9) to calculate the position of the tissue samples in the PCA plot as defined by the developmental series (Figure 4C). Based on their tissue expression pattern (9 tissues), clones were arbitrarily classified as either tissue-specific (ts) or constitutive (co). A clone n is considered tissue-specific if the difference between the lowest ($R_{min}(n)$) and highest ($R_{max}(n)$) expression ratio for the tissues under investigation is at least 3-fold. Tissue-specific clones were then scored for each tissue (t) as either maximally expressed (ts+) or not (ts-): ts+ if the expression ratio (R(n,t)) is at least 0.5 times the maximum expression ratio ($R_{max}(n)$) observed, else ts-.

Quantitative RT-PCR

Expression levels of genes were determined by real-time quantitative RT-PCR and the corresponding primers were designed using the Primer Express software (version 1.5, PE Applied BioSystems, CA, USA). Total RNA (1 µg) from each of the eight developmental

time points was treated with DNase I (Boehringer Mannheim) before undergoing reverse transcription, using random hexamers as primers and Multiscribe™ Reverse Transcriptase (Applied Biosystems) to generate a first strand cDNA template. Potato ubiquitin primers were used as a control and were based on the potato ubiquitin-ribosomal protein gene sequence (ubi3; L22576). Aliquots of 50 ng of cDNA for each time point were amplified with gene specific primers on the Perkin Elmer ABI Prism 7700 sequence detector in conjunction with the SYBR green PCR master mix kit (Applied Biosystems). Thermal cycling conditions were 10 min 50˚C, 5 min 95˚C followed by 40 cycles (15s at 95˚C, 1 min at 60˚C). Primer sequences: *ubi3* (L22576) forward primer 5'-TTCCGACACCATCGACAATGT-3', reverse primer 5'-CGACCATCCTCAAGCTGCTT-3'; GA2-oxidase homolog (BI176613), forward primer 5'-AGGCACAGAGTGATCGCAGAT-3', reverse primer 5'-TGGTGGCCCTCCAAAGTAAA-3'; *adr11-2* homolog (BE339784), forward primer 5'-CAAAGGCAACATGCCCAATT-3', reverse primer 5'-CCAAGAAGATCCACACAAGCC-3'; *arcA* homolog (BI177453), forward primer 5'-ACCATTCAGGACGGAGACTCA-3', reverse primer 5'-TGAAACGCACACACGAAACC-3'; GA2-oxidase homolog (BG096003), forward primer 5'-TCTCTGCAGGGAGTTACCGCT-3', reverse primer 5'-TTGTTGAAGACAAGTAGCTGAAGGA-3'. 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).

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Supplementary Material

The authors have supplied supplementary material containing all expression data presented in this paper available. Table S1: relative expression data (set A) of 1315 genes during eight tuber developmental stages (Figure 1, stages 1–8); Table S2: relative expression data (set B) of 1315 genes during six tuber developmental stages; Table S3: relative expression profiles of starch metabolism genes during potato tuber development (Figure 3); Table S4: relative gene expression and specificity of 1315 genes in a set of potato tissues.

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Chapter 3

StGA2ox1 is induced prior to stolon swelling and controls GA levels during potato tuber development

(*submitted*)

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Abstract

Potato tuber formation and growth is a complex process regulated by different environmental signals and plant hormones. In particular, the action of the plant hormone, gibberellin (GA), has been implicated in different aspects of potato tuber formation. GA 2-oxidase family members catalyze the conversion of biologically active GAs into inactive forms through 2β-hydroxylation. Here we report the analysis of transgenic potato plants either over-expressing or having reduced transcript levels of a potato GA 2-oxidase gene (*StGA2ox1*). *StGA2ox1* is up-regulated during the early stages of potato tuber development prior to visible swelling. 35S-over-expression transformants exhibit a dwarf phenotype, reduced stolon length and earlier *in vitro* tuberization. Transgenic plants with reduced expression levels of *StGA2ox1* showed normal plant growth, an altered stolon swelling phenotype and delayed *in vitro* tuberization. Tubers of silencing clones contain increased levels of GA_{20}, indicating altered gibberellin metabolism. We propose a role for StGA2ox1 in tuber development through the action of regulating GA levels at tuber formation, facilitating normal tuber development and growth.

Introduction

Potato plants have the capability to produce underground storage organs called tubers. In general, tuberization is promoted by short days (SD) with the degree of response largely dependent on genotype and physiological age of the plant (Ewing and Struik, 1992). Plants induced to tuberize produce a graft transmissible signal in the leaves that is transported basipetally to the growing stolon tip where it promotes tuberization (Gregory, 1956; Jackson *et al.*, 1998). The exact nature of this transmissible signal is still unknown but is most likely to be based on a mixture of both inducing and inhibiting types of signals (Ewing and Struik, 1992; Jackson, 1999; Martinez-Garcia *et al.*, 2001). Gibberellins (GAs) have long been implicated in playing a regulating role in potato stolon growth and tuber initiation (Booth, 1963; Kumar and Wareing, 1974; Ewing, 1987). Applications of biologically active GAs or inhibitors of GA biosynthesis have shown to either delay or promote tuber formation under tuber inducing conditions (Vreugdenhil and Struik, 1989; Jackson and Prat, 1996). Furthermore, several lines of evidence indicate the existence of cross-talk between day length perception and GA metabolism (Jackson *et al.*, 2000; Amador *et al.*, 2001; Chen *et al.*, 2003). In the last decade, significant progress has been made in identifying the genes involved in GA metabolism (for review see Lange, 1998; Hedden and Proebsting, 1999; Hedden and Phillips, 2000). In potato, Northern blot analysis of three isolated GA20-oxidase genes showed expression of all three genes in both stolon and tuber tissue (Carrera *et al.*, 1999), yet little is known about the corresponding enzyme activities in these tissues. Over-expression of *StGA20ox1* results in plants that show delayed tuber formation, whilst silencing clones are semi-dwarfed and tuberize earlier under short day conditions (Carrera *et al.*, 2000). Whether biologically active GAs or their precursors are part of the transmissible signal or play a role in the production, sensitivity or transport of tuber promoting or inhibiting signals, remains to

be resolved. The identification of novel genes regulating GA levels through transcriptional control or altering GA sensitivity in potato plants thereby controlling time point of tuberization such as, StBEL5 (Chen *et al.*, 2003), POTH1 (Rosin *et al.*, 2003), and PHOR1 (Amador *et al.*, 2001), indicates the existence of a complex mechanism for controlling GA levels during potato tuber formation. Another important piece of evidence, linking GAs to the regulation of tuber development, was the observation of a decrease in the levels of GA_1 within the subapical region of *in vitro* grown microtubers prior to visible swelling (Xu *et al.*, 1998a). When potato plants are induced to tuberize, stolon growth ceases and the reduction in the levels of GA is thought to result in the longitudinal reorientation of the cell microtubules and microfibrils, allowing lateral cell expansion and division (Shibaoka, 1993; Fujino *et al.*, 1995; Xu *et al.*, 1998b). The regulatory mechanism by which levels of bioactive GAs are reduced prior to stolon swelling is, however, still unknown.

In many plant species, bioactive GAs are inactivated through 2β-hydroxylation to produce inactive forms of GAs. This step is catalyzed by a member of the 2-oxoglutarate dependent dioxygenase family, GA 2-oxidase (Thomas *et al.*, 1999). GA 2-oxidase activity can also inactivate precursors of active GAs, like GA_{20} (Martin *et al.*, 1999). Similar to the GA 20-oxidase and GA 3-oxidase gene families, the GA 2-oxidase gene family consists of multiple members with often overlapping functions. In recent years, many GA 2-oxidase genes have been identified in various plant species and are considered to be important regulators of GA metabolism (Hedden, 2001).

In a previous study we found strong up-regulation of a potato GA 2-oxidase family member during the switch from long to short day conditions within the stolon tip (Kloosterman *et al.*, 2005). Here we present the isolation and functional analysis of a GA 2-oxidase family member, *StGA2ox1*. We propose a role for StGA2ox1 in regulating the GA levels during the switch from longitudinal stolon growth to radial swellings and subsequent tuber development.

Results

StGA2ox1 is a GA 2-oxidase family member highly expressed in tubers

Using a cDNA-microarray approach, we have identified a potato EST with homology to a GA 2-oxidase that shows increased expression levels during tuber development (Kloosterman *et al.*, 2005). Transcript levels increase significantly after the switch from long day to short days and an even larger increase was observed at tuber organogenesis after which transcript levels remain relatively stable as was confirmed by quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR). Full length sequence of the potato GA 2-oxidase was obtained by sequencing clone BI176613 derived from an *in vitro* grown microtuber cDNA-library (Ronning *et al.*, 2003). Sequence analysis revealed a complete open reading frame of 340 amino acids with a predicted molecular weight of 38 kD. The isolated potato gene described here, is the first GA 2-oxidase identified in potato and was named

StGA2ox1. The deduced amino acid sequence showed high levels of homology to other GA 2-oxidase genes isolated in a number of species (Figure 1). The highest sequence homologies to StGA2ox1 were found for GA 2-oxidases from *Nerium oleander*, NoGA2ox3 (AAT72916) 71.8%) and *Daucus carota*, DcGA2ox1 (BAD30038) (Mitsuhashi *et al.*, 2003), sharing respectively 71.8% and 57.1% of sequence similarity at the amino acid level. Analysis of the potato EST database provided a number of GA 2-oxidase homologues suggesting the likely existence of a multigene family in potato as has been found in other plant species.

Figure 1; Cluster analysis of amino acid sequence of identified members of the GA 2-oxidase enzyme class including StGA2ox1 (bold). Sequences were aligned using the AlignX program part of the Vector NTI suite (http://www.informaxinc.com). The phylogenetic tree is visualized with the Treeview program (http://rana.lbl.gov/EisenSoftware.htm). The following enzymes were included: *Arabidopsis thaliana* (At); AtGA2ox1(AJ132435), AtGA2ox2(AJ132436), AtGA2ox3(AJ132437), AtGA2ox4 (AAG51528), AtGA2ox6 (AAG00891), AtGA2ox7 (AAG50945), AtGA2ox8 (CAB79120). *Cucurbita maxima* (Cm); CmGA2ox1 (AJ315663). *Daucus carota* (Dc); DcGA2ox1 (BAD30038), DcGA2ox2 (BAD30039). *Hordeum vulgare* (Hv); HvGA2ox4 (AAT49062), HvGA2ox5 (AAT49063). *Lactuca sativa* (Ls); LsGA2ox1 (AB031206), LsGA2ox2 (AB031207). *Nerium oleander* (No); NoGA2ox1 (AY594291), NoGA2ox2 (AY594292), NoGA2ox3 (AY588978). *Nicotiana sylvestris* (Ns); NsGA2ox1 (AAO92303). *Nicotiana tabacum* (Nt); NtGA2ox1 (BAD17855), NtGA2ox2 (BAD17856). *Oryza sativa* (Os); OsGA2ox1 (BAB40934), OsGA2ox2 (BAC16751), OsGA2ox3 (BAC16752), OsGA2ox4 (AAU03107), OsGA2ox5 (BAC10398), OsGA2ox6 (CAE03751). *Phaseolus coccineus* (Pc); PcGA2ox1 (CAB41036). *Pisum sativum* (Ps); PsGA2ox1 (AAF08609), PsGA2ox2 (AAD45424). *Populus alba x Populus tremuloides* (Pta); PtaGA2ox1 (AAQ93035). *Spinacia oleracea* (So); SoGA2ox1 (AAN87571), SoGA2ox2 (AAN87572), SoGA2ox3 (AAX14674). *Vigna angularis* (Va); VaGA2oxA1 (AB181372), VaGA2oxA2 (AB181373), VaGA2oxB1 (AB181374), VaGA2oxB2 (AB181374), VaGA2oxB3 (AB181376).

Expression analysis of *StGA2ox1* in different potato tissues using qRT-PCR indicated high levels of expression in the tuber while minor expression was found in root, stem and stolon (Figure 2). The high expression found in young tubers suggests a specific role for StGA2ox1 in controlling GA levels within the tuber.

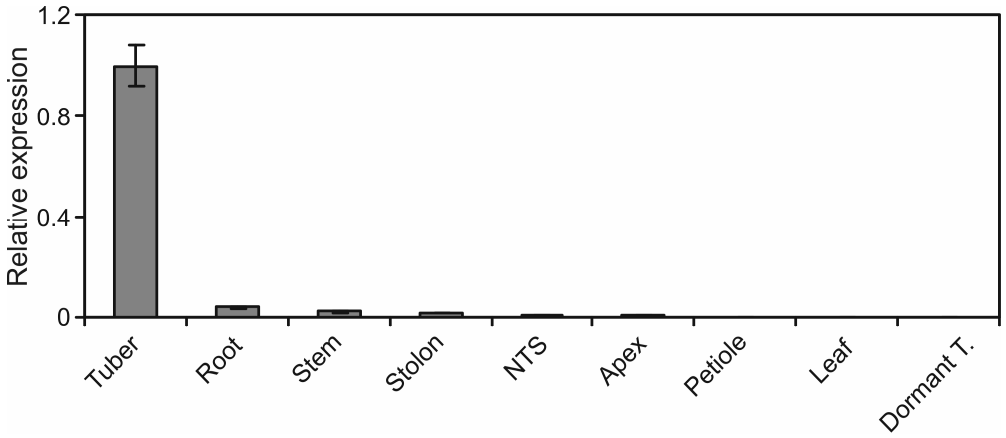

Figure 2; Comparison of the relative expression levels of *StGA2ox1* in the different tissues using qRT-PCR. Expression levels were calculated relative to the tissues in which expression level was highest (Tuber). Tissues studied include 3 week old tuber, root, stem, stolon, non-tuberizing stolon tip from LD plants (NTS), shoot apex, petiole, leaf and dormant tuber (stored for 4 weeks at 4˚C).

Over-expression and silencing of StGA2ox1 expression results in altered growth characteristics and tuber morphology

To further elucidate the function of StGA2ox1 we produced 35S-CaMV over-expression and silencing (RNAi) clones of potato *var*. Karnico. Forty-five independent silencing and nine over-expression clones were transferred to soil and grown in the greenhouse to check for altered expression of *StGA2ox1*. Transgenic clones showing the severest phenotypes were selected and after confirmation of the presence of at least one insertion of either construct, the transgenic plants were grown under controlled conditions in 10 replicates for further analysis. Plants were kept under long day conditions (16h light) for a period of 6 weeks after which the growth conditions were switched to short days (8h light) to promote tuber formation. Tuber induction in the Karnico variety is not under strict photoperiodic control and in our experiments we already observed some tuber formation in both transgenic and control plants under long day conditions. Within the individual replicates of each clone, plants did not tuberize synchronously and therefore we were not able to statistically determine a difference in time point of tuberization between the transgenic clones and control plants. However, several clear phenotypic differences were observed. Transgenic plants over-expressing *StGA2ox1* are semi-dwarfed and have smaller leaves (Figure 3A-B). These plants exhibited reduced stolon growth and formed small tuber initiations directly on the underground stem or developed from lateral stolon buds (Figure 3E). The formation of these small tuber incipients coincides with stolon initiation four weeks after planting but they do not continue to grow in size as would occur during normal tuber development. As in the

control plants, tuber development and further tuber growth was observed after six weeks of growth under long day conditions. Total tuber yield was reduced in the over-expression clones with plants on average producing either fewer or smaller tubers (Figure 3J-L). The silencing clones did not show marked changes in the aerial part of the plant (Figure 3C). However, a number of transgenic plants produced 'elongated' tuber swellings or had a higher degree of stolon branching in the underground parts (Figure 3F-I).

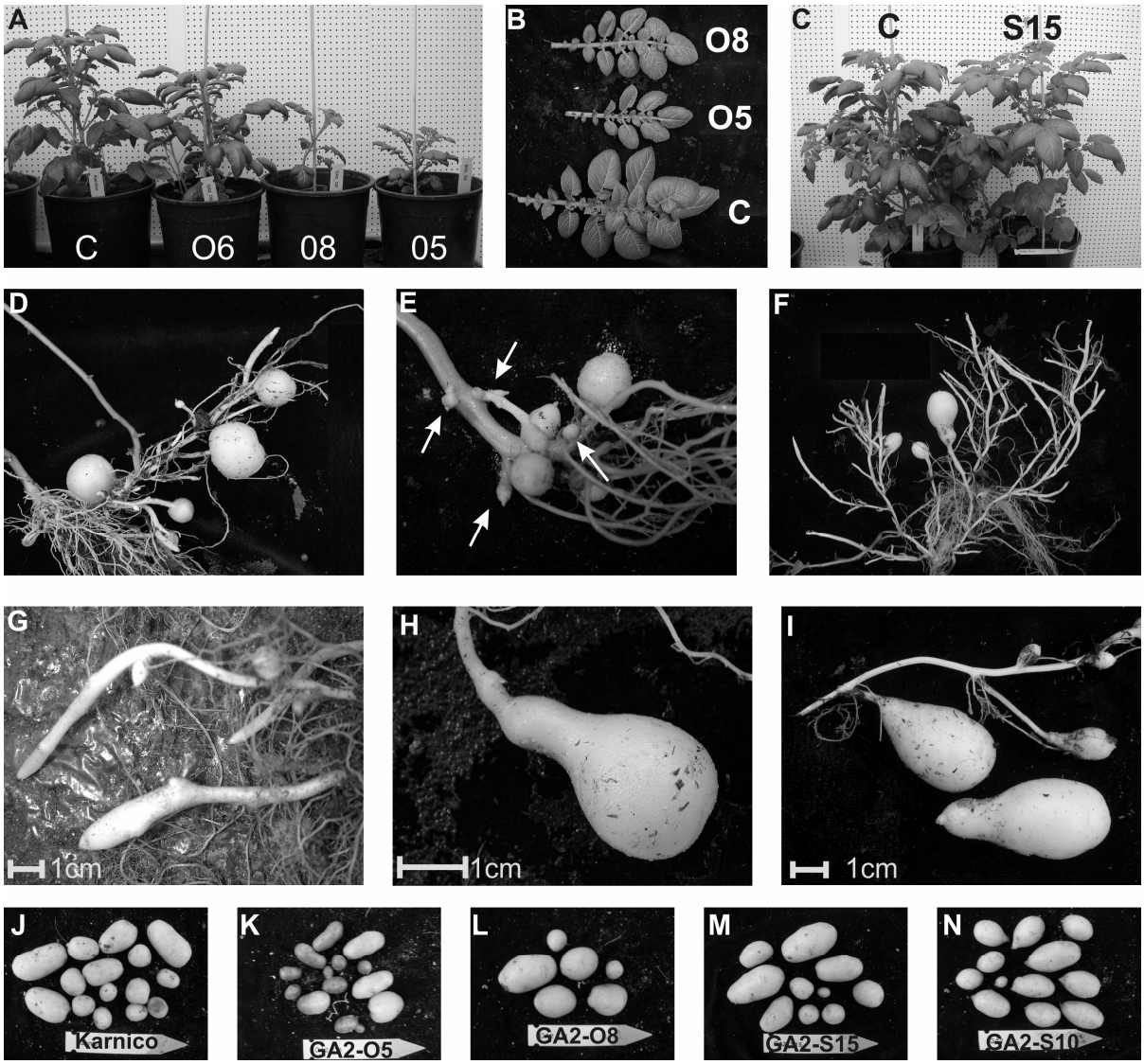

Figure 3; Observed phenotypes for StGA2ox1 over-expression clones O5, O6, O8 and silencing clones S9, S10, S15 and untransformed control plants (A-N). Photographs of 4 week old plants (A) and leaves (B) and six week old plants (C) of control and transgenic clones grown under controlled environmental conditions. Stolon growth in the untransformed control (D), Over-expression clone O5 (E) and in silencing clone S9 (F). Different developmental stages of the elongated tuber swelling phenotype (G-I). Tuber yield of control and transgenic plants harvested after the plants had completely died off (J-N).

The elongated swellings were most clearly visible during early stages of tuber development (Figure 3G). Secondary growth of these tubers reduces the elongated phenotype (Figure 3G-I). It is however important to note that the majority of mature tubers in the silencing clones

appear normal (Figure 3M-N). Total tuber yield and the number of tubers produced were not significantly altered in comparison to the controls (data not shown).

Expression levels of *StGA2ox1* in active sink tubers of the transgenic plants were determined using qRT-PCR and are plotted relative to the expression levels found in the tubers of the control plants (Figure 4). The transcript levels of *StGA2ox1* in over-expression clones O5 and O8 were increased up to 13- and 10-fold respectively, whilst in the silencing clones S10 and S15, transcript levels were reduced 3.9- and 3.6-fold respectively. Although the expression levels in the silencing clones are significantly reduced, low levels of *StGA2ox1* transcripts are still present and therefore some levels of StGA2ox1 activity are probably maintained throughout tuber growth. Tubers from both sets of transgenic plants do not differ in the duration of their dormancy period in comparison to the control tubers when stored at 20˚C (data not shown).

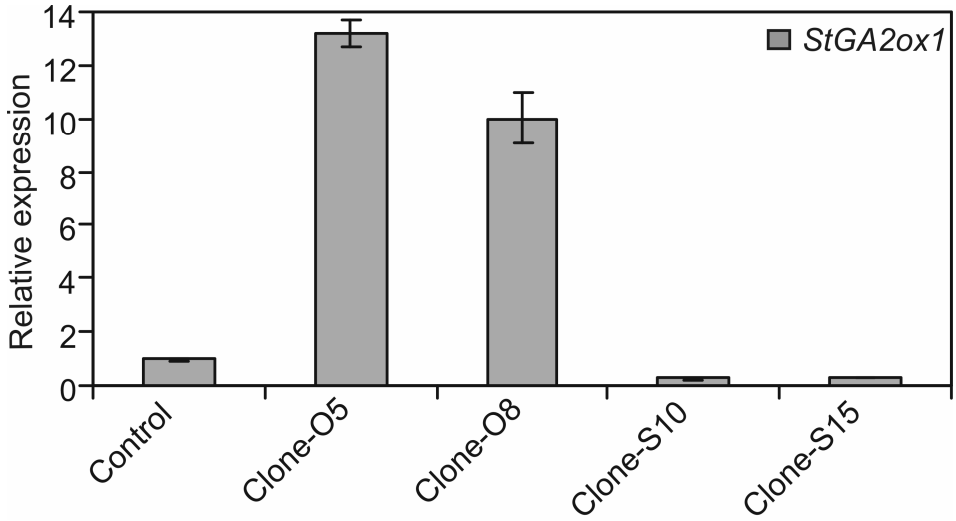

Figure 4; Fold-change in the expression level of *StGA2ox1* in tubers of the over-expression (O5, O8) and silencing clones (S10, S15) relative to the untransformed control. Error bars indicate the standard deviation between three independent measurements.

GA levels in transgenic tubers

GA levels were measured in active sink tubers of both over-expression and silencing clones and untransformed control plants in order to ascribe the observed phenotypes to altered GA metabolism (Table I). A dramatic increase of GA_{20} and a smaller but profound increase for both GA_1 and GA_8 were observed in the tubers of both silencing clones, S10 and S15. No significant differences were found for GA metabolites of the non 13-hydroxylation pathway (Table I: GA_{12}, GA_9, GA_{51}, GA_4 and GA_{34}) and therefore it was concluded that StGA2ox1 is primarily active on metabolites of the early 13-hydroxylation pathway (Table I; GA_{53}, GA_{20}, GA_{29}, GA_1 and GA_8). Since GA_{20}, GA_1 and GA_8 levels are all increased in the silencing clones, it appears that GA turnover in this part of the pathway is elevated due to the increased availability of the GA_{20} precursor (Figure 5). Levels of GA_{29} do not change significantly in either silencing clone. Interestingly, no significant difference in GA content could be

observed in the over-expression clones (O5 and O8) in comparison to control plants (Table I). A small and consistent reduction in the levels of GA_{29} was observed in both over-expression clones though its significance remains unclear. The levels of $GA_{5,}$ GA_3 and GA_{29}-catabolite (Figure 5) were not measured in the transgenic plants and therefore we were unable to determine if the endogenous levels in these parts of the GA pathway had changed.

Table I: Measured levels of GAs in potato tubers from untransformed control plants and *StGA2ox1* over-expression and silencing clones

	GA-levels (ng/g FW)				
	Control	Clone-O5	Clone-O8	Clone-S10	Clone-S15
Non 13-hydroxylation pathway					
GA_{12}	0,03	0	0,05	0,02	0
GA_9	0,01	0	0,02	0,01	0
GA_{51}	0,04	0,01	0,03	0,03	0
GA_4	0,06	0,16	0,05	0,04	0,05
GA_{34}	0,01	0,01	0,01	0,01	0,02
13-hydroxylation pathway					
GA_{53}	0,04	0,01	0,01	0,01	0,01
GA_{20}	0,01	0	0	**0,49**	**0,33**
GA_{29}	0,27	0,13	0,15	0,23	0,24
GA_1	0,01	0,02	0,04	**0,09**	**0,10**
GA_8	0,03	0,01	0,02	**0,23**	**0,21**

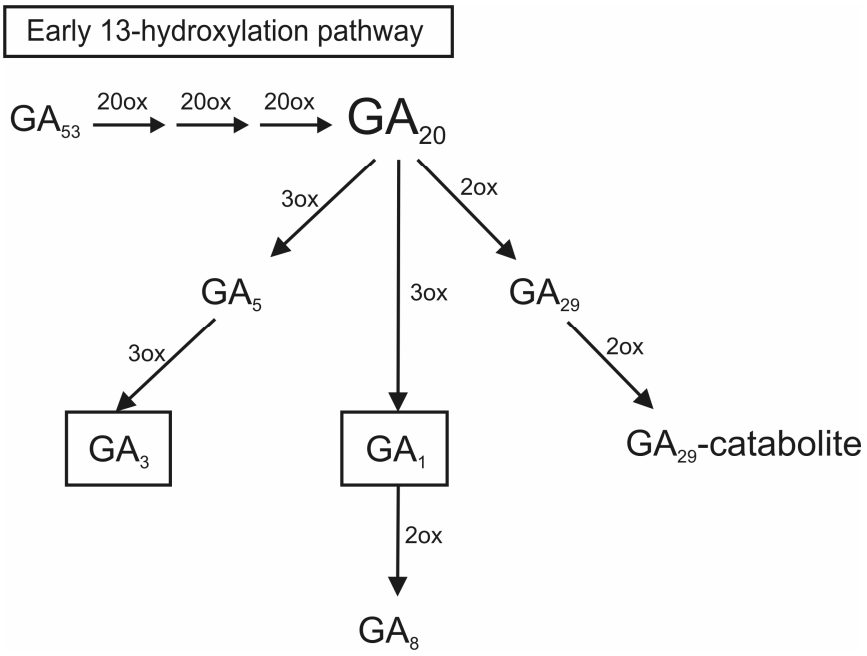

Figure 5; Schematic overview of the major genes involved in the 13-hydroxylation pathway in plants. Steps catalyzed by the different GA-oxidase genes are indicated; GA20-oxidase (20ox), GA3-oxidase (3ox), GA 2-oxidase (2ox). Biologically active GAs are boxed.

Expression levels of StGA2ox1 and other GA metabolism genes are altered in the transgenic plants

In order to further elucidate gibberellin metabolism in the tuber we looked at the expression levels of other GA metabolism genes. First of all, the expression levels of known potato GA 20-oxidase and GA 3-oxidase genes were measured during tuber development (Figure 6A). We found a similar expression profile for *StGA20ox1* as was observed for *StGA2ox1* during the eight developmental stages (Figure 6B) with the strongest increase of expression levels occurring in the swelling stage (Figure 6A; Stage 3). *StGA20ox3* transcript levels are also increased at tuber organogenesis but the expression profile is characterized by a more gradual increase reaching peak levels at stage 6. In potato, two GA 3β-hydroxylase genes have been isolated from sprouts, *StGA3ox1* (AAK91507) and *StGA3ox2* (AAK91506), but little is known about their expression levels or activity within the plant. With qRT-PCR we were able to detect expression of one of the GA 3β-hydroxylase genes (*StGA3ox2*) in the non-tuberizing stolon tips and early stages of tuber development (Figure 6B).
After the switch from long day to short day conditions (Figure 6A; stage 1 and 2), transcript levels of *StGA3ox2* decrease until no transcripts could be detected from tuber stage 5 onwards. The strong down-regulation of *StGA3ox2* indicates a reduction of GA synthesis within the tissues studied. Like *StGA2ox1* and *StGA20ox1*, expression levels of *StGA3ox2* already change after the switch from long day to short day conditions prior to visible swelling and with the strongest change in expression levels occurring during stolon swelling (Figure 6A; stage 3). The strong induction of gene expression of *StGA2ox1, StGA20ox1, StGA20ox3* and the down regulation of *StGA3ox2* during tuber formation, indicates the existence of a complex regulatory mechanism of GA metabolism at the transcriptional level.

To identify possible feedback or feedforward control mechanisms, we analyzed the identified GA metabolism genes in the transgenic clones. In line with our previous results, we were not able to detect any transcripts of *StGA3ox1* and *StGA3ox2* within tubers > 0.8cm. However, we did observe differential expression of the GA 20-oxidase genes in the transgenic plants. A significant reduction of *StGA20ox1* and *StGA20ox3* expression levels in the silencing clones was observed (Figure 7). In particular *StGA20ox3* shows a strong decrease in silencing clones S10 and S15 compared to the control, 22.9- and 22.6-fold respectively. Interestingly, we could not detect a significant difference in the expression levels of the GA 20-oxidase genes in the over-expression clones. The data presented in Figure 7 show that down-regulation of *StGA2ox1* transcript levels in potato tubers, results in down-regulation of transcript levels of specifically *StGA20ox3* and, to a lesser extent, *StGA20ox1*.

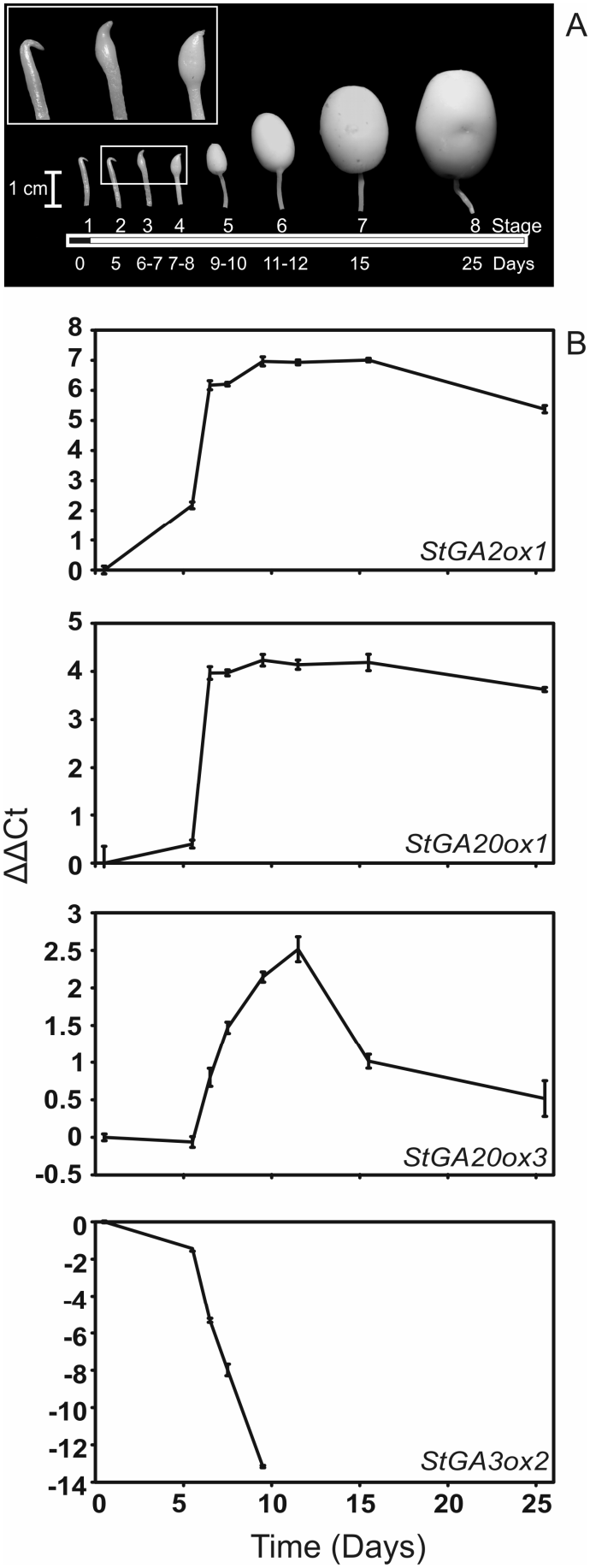

Figure 6; Quantitative RT-PCR measuring gene expression of a subset of GA metabolism genes during potato tuber development. RNA derived from eight tuber developmental stages harvested over a period of 25 days after a switch from a 16 to a 8h light period (A) (from Kloosterman *et al.*, 2005; © Blackwell publishing, reprinted with permission). Expression levels of GA metabolism of the eight developmental stages were calculated relative to potato *ubi3* gene and the first harvest (Stage 1) (B). Error bars show the standard error of the mean of three measurements. Genes measured include; *StGA2ox1*; *StGA20ox1*; *StGA20ox3* and *StGA3ox2*.

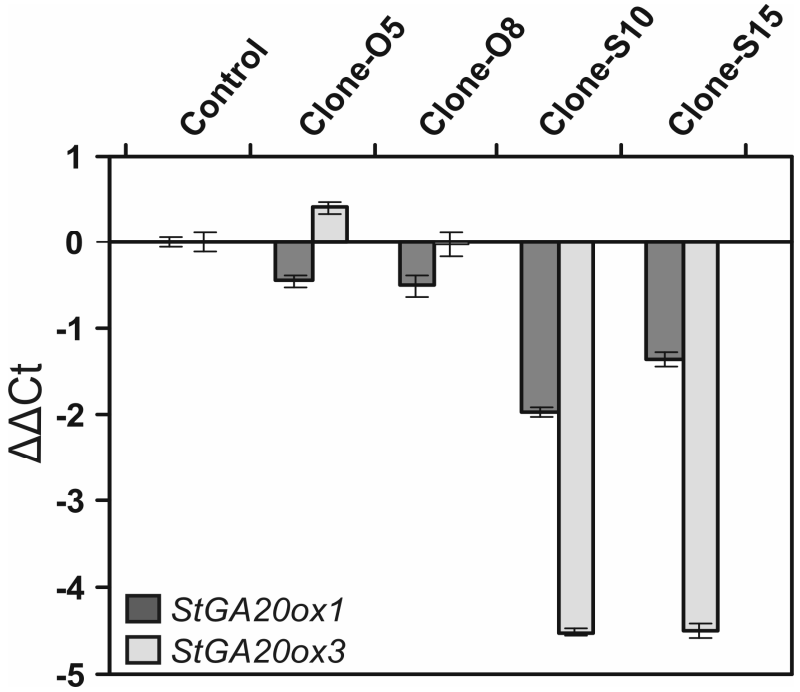

Figure 7; Impact of altered transcript levels of *StGA2ox1* on the expression of GA20-oxidase genes, *StGA20ox1* and *StGA20ox3*. Expression levels of *StGA20ox1* and *StGA20ox3* were measured in sink tubers of over-expression clones (O5 and O8) and the silencing clones (S10 and S15). Expression levels in the transgenic plants were calculated relative to the expression levels of the potato *ubi3* gene and the untransformed control. Error bars indicate the standard deviation between three independent measurements.

In vitro tuberization of StGA2ox1 transgenic plants

Since the phenotype produced by the silencing clones was partially lost due to secondary growth of the tuber, we decided to study early tuberization events using an *in vitro* tuberization assay, in which secondary growth is absent. Interestingly, in our *in vitro* experiment a clear increase in the induction of tuber formation was found in the over-expression clones and a delay in tuber formation in the silencing clones (Figure 8). Already one week after incubation in the dark, 95.1% and 84.6 % of the nodal stem cuttings from over-expression clones O5 and O8 had formed microtubers respectively, whilst in silencing clone S10, tuber formation was still absent. Only after three weeks a strong increase in the amount of tuber formation in both silencing clones was observed. In our soil grown plants we were not able to detect a significant difference in time point of tuber initiation although early small tuber incipients were found in the over-expression clones.

Besides shift in time point of tuberization, morphologically significant differences between the individual transgenic clones and control plants could be observed (Figure 9). In the over-expression clones, a large percentage of the formed tubers (78.9%) are sessile where stolon growth is largely inhibited. The silencing clones however, have a much larger percentage of lateral tuber formation (43.1%) and produce a low number of sessile tubers (7.6%) in comparison to the control plants. Furthermore, around 10% of the apical tubers,

formed in the silencing clones, exhibited an elongated tuber shape very similar to that found in *in vivo* grown plants (Figure 10A-D). Whilst in the control plantlets, tubers generally have a round shape, in which the first and second node are incorporated in the radial swelling, the silencing clones often have an elongated phenotype between the first and second node of the *in vitro* tuber. In addition, lateral tubers were sometimes formed directly from the tuber eyes (Figure 10B). In an additional experiment we applied different concentrations of GA_3 and the GA biosynthesis inhibitor, ancymidol, to the tuber inducing medium (Figure 9). The control plants supplemented with 0.1μM GA_3 produced fewer sessile tubers (17.0%) in comparison to the normal tuber inducing medium (47.0%) and had an increased number of lateral tubers, indicating lateral tuber formation is at least in part regulated by GA levels. Reduction in the number of sessile tubers is further enhanced in medium supplemented with 1.0 μM GA_3.

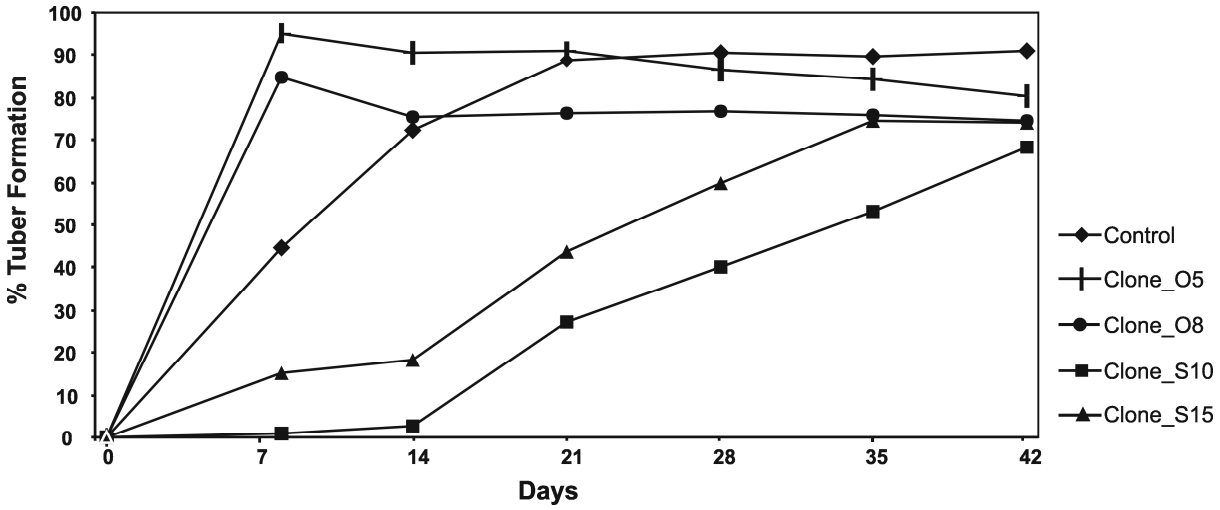

Figure 8; In vitro tuberization of single node cuttings from untransformed control plants (C; ♦) and *StGA2ox1* over-expression clones (O5; ▌ and O8; ●) and silencing clones (S10; ■ and S15; ▲). Relative percentage of tuber formation was scored on a weekly basis.

Supplementing the over-expression clone O8 with 0.1 μM GA_3 does not seem to have a large effect on the number or type of sessile tubers formed, 78.9% without and 72.1% with 0.1 μM GA_3 supplementation, respectively. However, when adding 1.0μM GA_3 the percentage of sessile tubers formed decreased significantly (42.5%) together with an increase in longitudinal stolon growth. These findings are consistent with the inhibiting effect of GA_3 on tuber formation in potato. Most likely, due to the increased inactivation of GA in the over-expression clones, higher levels of GA_3 supplementation are needed to cause a significant effect on tuber formation. In silencing clone S15, a reduction in the percentage of apical tubers was observed relative to the amount of GA_3 supplied but did not lead to an increase in lateral tuber formation as was found for the control. In conjunction with these observations, when supplementing the tuber inducing medium with 0.5 μM ancymidol, a GA synthesis inhibitor, stolon growth is largely absent in the over-expression clones and control, while in the silencing clones the effect is much smaller.

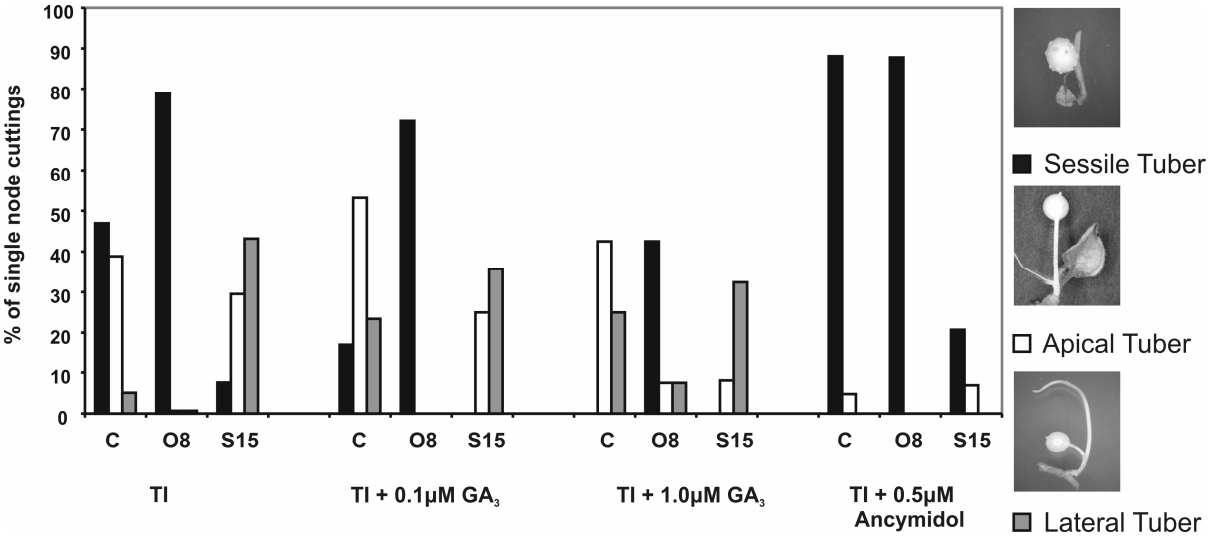

Figure 9; Distribution of the type of tubers scored in an in vitro tuberization experiment of untransformed control plants (C), over-expression clone (O8) and silencing clone (S15). Single node cuttings were harvested from 4 weeks old in vitro grown plantlets and incubated in the dark (18˚C) on tuber inducing medium (TI) or tuber inducing medium supplemented with either 0,1 µM GA_3, 1.0 µM GA_3 or 0,5 µM ancymidol. Images on the right panel illustrate the type of tubers that were scored.

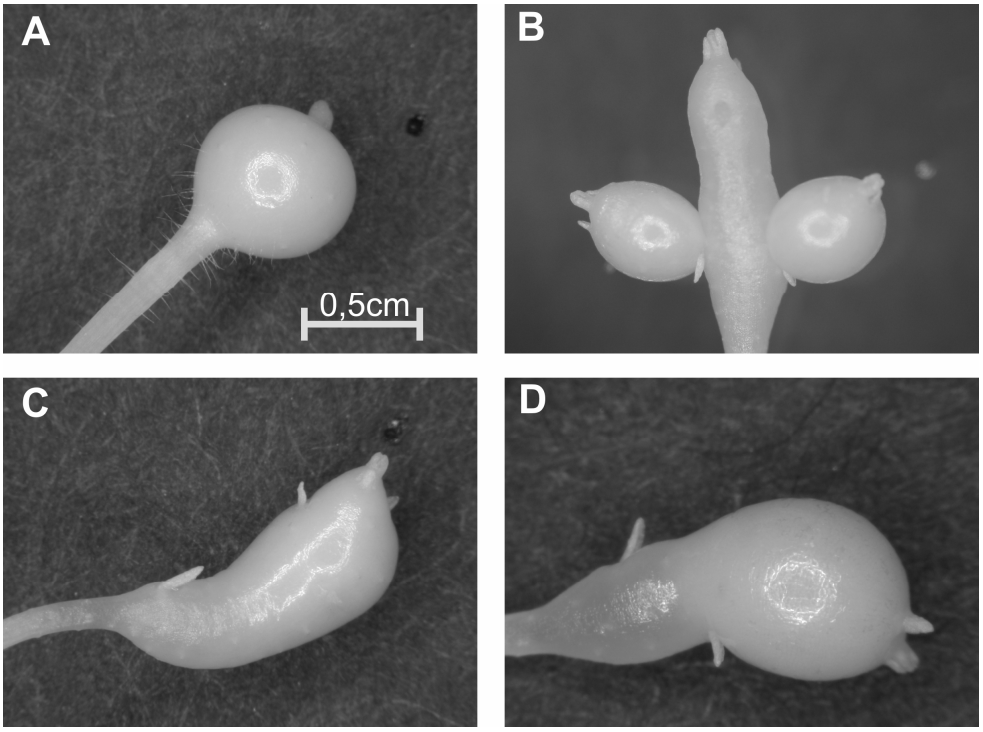

Figure 10; Photographs of collected *in vitro* grown microtubers from untransformed control (A) and *StGA2ox1* silencing clones showing elongated swellings; S9 (B), S10 (C), and S15 (D). Photographs were taken under binocular, relative scale is indicated with bar of 0.5cm.

If GA synthesis in the stolon is absent due to the presence of an inhibitor, GA levels present within the nodal stem cuttings at time point of harvest appear to be more long-lived in the silencing clones in comparison to the over-expression and control plantlets possibly due to a reduction of GA inactivation.

Taken together these findings suggest that GA levels are reduced in the over-expression clones resulting in inhibition of stolon growth and formation of sessile tubers, whilst in the silencing clones GA levels remain high within the stolon tip delaying tuber formation and promoting lateral stolon growth and subsequent lateral tuber formation.

Discussion

Over-expression and silencing of StGA2ox1 results in altered tuber formation

We have identified a GA 2-oxidase homolog in potato which we named *StGA2ox1*. *StGA2ox1* is lowly expressed during stolon growth and strongly up-regulated during early potato tuber development, indicating a role in regulating GA levels at tuber organogenesis. Over-expression of *StGA2ox1* under control of the 35S-CaMV promoter results in semi-dwarf plants with smaller leaves and reduced stolon growth. Reduced *StGA2ox1* transcript levels in the silencing clones does not lead to an altered phenotype in the aerial parts of the plants confirming the specificity of *StGA2ox1* expression within the tuber. Although we could not detect a significant difference in the time point of tuberization between the control plants and the *StGA2ox1* over-expression and silencing clones in the *in vivo* experiment, a clear difference was observed in the *in vitro* tuberization experiment (Figure 8). Either a strong induction or delay in the rate of *in vitro* tuberization was observed for the over-expression and silencing clones respectively, consistent with altered GA content (Vreugdenhil and Struik, 1989; Jackson and Prat, 1996; Xu *et al.*, 1998a). The difference in the time point of tuber formation between *in vivo* and *in vitro* tuberization can be explained by the nature of the tuber induction signal in both systems. Under *in vitro* conditions, high sucrose levels are thought to be the major signalling component in tuber formation. It has been shown that sucrose concentration correlates negatively with GA levels in the stolon tip (Xu *et al.*, 1998a). Under *in vivo* conditions there is evidence for the presence of at least two independent tuber inducing pathways, a photoperiod-dependent pathway and a GA dependent pathway (Martinez-Garcia *et al.*, 2001). Furthermore, the production of the tuber inducing signal of *in vivo* grown plants is also dependent on plant size and maturity and other regulatory signals (Ewing and Struik, 1992). Hence, under *in vitro* conditions the time point of tuber formation appears to rely primarily on sucrose/GA interactions within the stolon tip (Xu *et al.*, 1998a) whilst under *in vivo* conditions additional factors are required to induce tuber formation. In this respect, it is interesting to note the finding of small tuber incipients *in vivo* in the over-expression clones which remain relatively small until secondary growth is induced (Figure 3E). These small ‘tubers’ appear to be thickened stolons that are inhibited in their normal tuber growth pattern due to lack of a specific growth signal (i.e. tuber inducing signal) similar to *in vitro* grown microtubers lacking secondary growth.

It has been well documented that GA levels promote longitudinal cell division and elongation within plants by affecting the orientation of microtubules and microfibrils (Shibaoka, 1993; Fujino *et al.*, 1995; Sanz *et al.*, 1996). Xu *et al*., (1998b) found that prior to *in vitro* tuber formation, GA_1 levels are strongly reduced, thereby potentially inducing a reorientation of the plane of cell division and expansion, resulting in radial swelling of the subapical part of the stolon. Over-expression of *StGA2ox1* appears to result in lower levels of bioactive GAs within the stolon tip reducing longitudinal stolon growth. Furthermore, the reduced GA levels in these transgenic plants may cause reorientation of the cell microtubules and microfibrils in an early stage of stolon initiation resulting in the small tuber incipients (Figure 3E). In the *in vitro* system longitudinal stolon growth is largely absent in the over-expression clones, resulting in the early formation of sessile tubers. Interestingly, the percentage of lateral tuber formation was significantly increased in the silencing clones (Figure 9), indicating a possible gradient of biologically active GAs within the stolon. It has been suggested that concentrations of GA_1 vary throughout the stolon, with the highest concentration located in the stolon tip (Jackson, 1999).

In the *StGA2ox1* silencing clones, elongated tuber swellings were observed at low frequency in both tuberization experiments. By lowering the ability to inactivate biologically active GAs, the reduction of GA levels prior to tuber formation might be insufficient, resulting in only a partial reorientation of the plane of cell division and expansion. A partial or dispersed reorientation may explain the observed elongated swelling morphology (Figure 3G-I and Figure 10B-D). During secondary tuber growth, cell divisions are randomly orientated in the perimedullary region often masking the initial elongated swelling phenotype. These findings clearly state an important role for StGA2ox1 in regulating potato tuber initiation.

Reduced transcript levels of StGA2ox1 results in increased levels of GA_{20}, GA_1 and GA_8

In order to infer catalytic activity of StGA2ox1, we measured a range of GA metabolites. Surprisingly, we did not find a significant increase or reduction of the studied GAs in the tubers of the over-expression clones. However, the phenotypes of these plants, semi-dwarfed, reduced stolon growth and earlier *in vitro* tuberization, are consistent with a reduction in GA content indicating that very small changes in GA homeostasis may already be sufficient to alter plant morphology. In the silencing clones a large increase of GA_{20} and a smaller but profound increase of GA_1 and GA_8 were observed. The turnover of GA_{20} within the tubers appears to be reduced in the silencing clones thereby creating an increased pool of GA_{20}. The increased availability of GA_{20} as a substrate, most likely results in an increased flux towards the synthesis of both GA_1 and GA_8. Altered regulation of GAs downstream of GA_{20} may be indirectly responsible for the increase of GA_{20} in tubers of the silencing clones. For example, the oxidation of GA_{29} to produce the GA_{29}-catabolite has been shown to be catalyzed by a GA 2-oxidase in pea shoots (Lester *et al.*, 1999; Martin *et al.*, 1999). Similarly, in maize shoots it was shown that GA_3 can be synthesized from GA_{20} via GA_5 (Fujioka *et al.*, 1990; Spray *et al.*, 1996). Abdala *et al.*, (2002) measured significant levels of

GA_3 in potato foliage, root, stolon and tuber. However, there is no data available on changing GA_3 levels during the transition of a stolon into a tuber as was reported for GA_1 levels (Xu *et al.*, 1998a). Additional experiments will have to be done in order to establish a regulating role for GA_3 in potato tuber development. Some evidence for a correlation between StGA2ox1 activity and GA_3 levels comes from our *in vitro* tuberization experiment, where transgenic plants over-expressing *StGA2ox1* grown on tuber inducing medium supplemented with GA_3, show an altered response in comparison to untransformed controls (Figure 9). A much larger concentration of GA_3 (1.0 μM) is needed to promote stolon growth in the over-expression clones in comparison to the control. Based on the quantified GA levels within the tubers, the reaction(s) that are catalyzed by StGA2ox1 in potato tubers remains obscure. The GA 2-oxidase gene family in general consists of multiple members which are often functionally redundant (Hedden and Proebsting, 1999; Thomas *et al.*, 1999), and therefore we can not exclude complementation of StGA2ox1 activity by a different family member in the transgenic plants.

Transcriptional control of potato tuber development

The detected increase of $GA_{20,}$ within tubers of the silencing clones, coincides with a decrease in the transcription levels of GA_{20} synthesis genes, *StGA20ox1* and *StGA20ox3* (Figure 7). Both GA20-oxidase genes have been shown to be under control of negative feedback regulation by biologically active GAs in potato (Carrera *et al.*, 1999). Whether the increase of GA_1 levels in the silencing clones could account for the observed decrease in expression levels of both GA 20-oxidase genes through negative feedback regulation remains to be seen. Unlike *StGA20ox1*, *StGA20ox3* is primarily expressed in underground tissues (Carrera *et al.*, 1999) and therefore may play a more prominent role in regulating GA levels within these tissues. The apparent contradiction of reduced expression of GA_{20} synthesis genes, and increased GA_{20} levels in the silencing clones, leads to the speculation that the transport of GA precursors, such as GA_{20}, from the leaves down to the stolon tip and growing tuber, may also be a regulatory component of GA_{20} content in potato tubers.

We also examined the expression levels of genes responsible for the last steps of GA metabolism during the early tuber development stages (Figure 6). Interestingly, we observed a strong decrease in expression levels of *StGA3ox2* prior to tuber formation. A decrease in GA biosynthesis would lower the levels of active GAs in the stolon tip thereby inhibiting longitudinal growth. Consequently, lowered levels of active GAs at tuber organogenesis could explain the observed increase of both *StGA20ox1* and *StGA20ox3* expression due to negative feedback regulation. The strong increase of *StGA2ox1* transcript levels prior to tuber formation raises the question whether there is a common mechanism controlling the different expression profiles found for the GA metabolism genes described here. When decapitating pea shoots, the reduction of auxin levels throughout the plant leads to a decrease of *PsGA3ox1* expression and an increase of *PsGA2ox1* expression levels (Ross *et al.*, 2000). The effects can be opposed by IAA application indicating a regulatory role in GA metabolism in pea. These findings may support a similar role for IAA in regulating *StGA3ox2* and

StGA2ox1 expression levels during potato tuber development. In addition, the observed expression changes in the stolons after the switch to short day conditions, implies that transcriptional regulation of *StGA2ox1*, *StGA20ox1* and *StGA3ox2* could be indirectly controlled by photoperiod. Overall GA 'activity' in the leaves was shown to decrease when leaves were exposed to short days (Kumar and Wareing, 1974). However, the mechanism by which day length perception in the leaves translates to GA metabolism or sensitivity in the stolon is still under investigation.

An additional regulatory mechanism of GA metabolism genes in potato comes from the finding of regulating StGA20ox1 promoter activity in potato through the binding of homeobox genes, StBEL5 and POTH1 (Chen *et al.*, 2003; Chen *et al.*, 2004). In another study, a MADS-box transcription factor, AGAMOUS-like 15, has been shown to bind to the regulatory region of *AtGA2ox6*, controlling its expression levels (Wang *et al.*, 2004). Whether expression levels of *StGA2ox1* and other GA metabolism genes in potato plants are also in part regulated through the binding of similar types of regulatory proteins still has to be investigated. The data presented in this paper on the expression of *StGA2ox1* and other GA metabolism genes during potato tuber development, provides novel insights into GA-regulated potato tuber formation at the organ level. From our expression studies in transgenic plants, it is clear that StGA2ox1 fulfils a central role in the transition from longitudinal stolon growth to tuber initiation by regulating GA levels.

Experimental procedures

Cloning of StGA2ox1 and sequence analysis

The sequence of the potato GA 2-oxidase was obtained by sequencing cDNA clone BI176613 deriving from an *in vitro* grown microtuber EST library (*cv.* Bintje). Sequence analysis revealed that the cDNA was full length and coded for a protein of 340 amino acids in size and was named StGA2ox1. The complete coding sequence of StGA2ox1 was amplified by PCR (Forward primer 5'-CACCTATGGTTGTTTTGTCTCA-3' and Reverse primer 5'-TGTCATGATTGAGCATTCT-3'). Forward primer contains a 5'-CACC partial overhang to facilitate cloning of the PCR product in pENTR/SD/D-TOPO vector (Invitrogen, Carlsbad, CA). *StGA2ox1* was further sub cloned by a LR recombination reaction into Gateway™ plant transformation destination vector pk7WG2 and pk7GWIWG2 (Plant Systems Biology, University of Ghent, Belgium) (Karimi *et al.*, 2002). The two resulting expression constructs were named pk7GA2ox1_O and pk7GAox1_S. pk7GA2ox1_O expression results in heterologous expression of *StGA2ox1* gene under control of the 35S-CaMV promoter. The pk7GA2ox1_S expression construct consists of a full length *StGA2ox1* inverted repeat harbouring an intron behind the 35S-CaMV promoter that when expressed produces a hairpin structure resulting in post transcriptional gene silencing (PTGS).

Plant Transformation and regeneration

pk7GA2ox1_O and pk7GAox1_S were transformed into *Agrobacterium tumefaciens* strain *Agl0* using electroporation. *In vitro* shoots of the *Solanum tuberosum* cv. Karnico were used for *A. tumefaciens* mediated transformation (Visser *et al.*, 1991). After regeneration of *in vitro* shoots on selective kanamycin MS medium (100 mg/L) (Murashige and Skoog, 1962), the shoots were transferred to the greenhouse to produce mature plants. From these transgenic plants three strong over-expression and silencing clones were selected for further analysis. Integration of at least one copy of either construct in the selected transgenic plants was confirmed by Southern blotting using part of the 35S-CaMV promoter as probe. Plants regenerated from untransformed *in vitro* shoots were used as wild type controls.

Plant material growth conditions and tuber harvest

Ten replicates of the *in vitro* control plants and of the three selected over-expression and silencing clones were transferred to soil and grown in a climate chamber (16 h light 20˚C and 8h dark at 18˚C) for a period of 6 weeks after which plants were transferred to short-day conditions (8h light and 16h dark period). Tubers from two repeats of each plant were harvested 6 weeks after the switch to short days and immediately frozen in liquid nitrogen. Frozen tubers were ground into a fine powder and used for expression studies and GA measurements. Remaining plants were left to senescence and die after which number of tubers and total tuber weight was determined from at least three repeats from each transgenic clone and control plants.

RNA isolation and Quantitative RT-PCR

For expression studies within the different transgenic clones and control plants, tRNA was isolated from tubers as described by Bachem *et al.*, (1996). For expression studies of the GA-oxidase genes during the potato tuber developmental time series (Figure 6A) and in the different potato tissues, total RNA was used from a previous study (Kloosterman *et al.*, 2005). One additional tissue sample was included, namely the shoot apex, and was harvested from 8 week old greenhouse grown plants (*S. tuberosum* cv. Karnico). Relative Expression levels of genes were determined by qRT-PCR on a Perkin Elmer Abi Prism 7700 Sequence detector (Perkin Elmer, CA, USA) following the protocol described in 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: *StGA2ox1* forward primer 5'-AGGCACAGAGTGATCGCAGAT-3', reverse primer 5'-TGGTGGCCCTCCAAAGTAAA-3'; *StGA20ox1* (CAC13036) forward primer 5'-CGGCCCAACAAGCATCTAAG-3', reverse primer 5'-AAGCCATGACTCCGACACG-3'; *StGA20ox3* (CAC13038) forward primer 5'-GCAATGCCATGAGCACCC-3', reverse primer 5'-

GGCTCAATCCCAAAAGTTCCA-3'. *StGA3ox2* (AAK91506) forward primer 5'-AGCTCATGTGGTCCGAAGGA-3'; reverse primer 5'-CGGACAAGCCGGGTAAGAAT-3'; *ubi3* (L22576) forward primer, 5'-TCCGACACCATCGACAATGT-3', reverse primer 5'-CGACCATCCTCAAGCTGCTT-3'.

GA measurements

For quantitative determination of endogenous GAs in tubers of the transgenic clones (O5, O8, S10 and S15) and untransformed controls (C), tubers from three independent replicates were harvested from 12 week old plants, mixed and immediately frozen in liquid nitrogen before being ground into a fine powder. Five gram of fresh weight tubers of each sample were spiked with 16, 17-d2-GA standards (5 ng each; from Professor L.Mander, Canberra, Australia). Samples ware extracted, purified, derivatized, and analyzed by gas chromatography mass spectrometry using selected ion monitoring as described elsewhere (Lange *et al.*, 2005).

In vitro tuberization assay

A modified version of in the *in vitro* tuberization method described by Hendriks *et al.*, (1991) was used for microtuber production. Instead of using *in vivo* grown plants grown for single node harvest, *in vitro* plantlets were used. The upper three nodal stem sections containing a single axillary bud from 4 week old *in vitro* grown plantlets were harvested from two independent over-expression and silencing clones and control plants. Over 200 stem sections for each clone were put directly on the tuber inducing medium and incubated in the dark (18˚C). Tuber inducing medium consists of modified Murashige and Skoog medium (Murashige and Skoog, 1962), containing 1/10 part of the standard amount of KNO_3 and NH_4NO_3, 8% w/v sucrose and 0.8% (w/v) agar with a final pH of 5.8. Tuber formation was scored weekly over a period of six weeks. In addition, around 50 stem sections were incubated on tuber inducing medium supplemented with either 0.1 μM GA_3, 1.0 μM GA_3 or 1.0 μM ancymidol and scored for tuberization. As a non-tuberizing control, plantlets were incubated on tuber inducing medium containing 1% (w/v) of sucrose.

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Chapter 4

Isolation and characterization of a novel potato *AUXIN/INDOLE-3-ACETIC ACID* family member (*StIAA2*) that is involved in petiole hyponasty and shoot morphogenesis

(*submitted*)

B. Kloosterman, R.G.F. Visser, C.W.B. Bachem

Abstract

Auxin/indole-3-acetid acid (Aux/IAA) proteins are short-lived transcriptional regulators that mediate their response through interaction with Auxin-response factors (ARFs). Although 29 Aux/IAA proteins have been identified in *Arabidopsis thaliana*, their individual functions are still poorly understood and are largely defined by observed growth defects in gain-of-function mutant alleles. Here we present the isolation and characterization of a novel Aux/IAA protein in potato (*Solanum tuberosum*) that is named StIAA2. Down regulation of *StIAA2* expression results in distinctive phenotypes that include, increased plant height, petiole hyponasty and extreme curvature of growing leaf primordia in the shoot apex. Gene expression analysis of transgenic plants with reduced *StIAA2* transcript levels resulted in the identification of a number of genes with altered expression profiles, including another member of the Aux/IAA gene family (*StIAA*). The phenotypes that were observed in the *StIAA2* suppression clones can be associated with both common as well as unique functional roles among Aux/IAA family members indicating the importance of analyzing Aux/IAA expression in different plant species.

Introduction

It is well known that Auxins, mainly represented by indole-3-acetic acid (IAA), are important in plant growth and developmental processes through the regulation of auxin-responsive gene expression (Theologis, 1989). In recent years, the regulatory components of auxin signalling have become more evident and have revealed the existence of a highly complex system of both early and late auxin responses (reviewed in Guilfoyle *et al.*, 1998a; Hagen and Guilfoyle, 2002; Woodward and Bartel, 2005). Several genes involved in the regulation of auxin dependent transcription have been identified, in which primary roles have been given to the auxin responsive factors (ARF) (Ulmasov *et al.*, 1997) and Aux/IAA protein family members (Abel *et al.*, 1994). ARF proteins can function as either transcriptional activators or repressors that can bind to auxin-responsive elements (AuxREs) found in promoters of auxin-responsive genes through a specific DNA binding domain (Ulmasov *et al.*, 1999). The Aux/IAA proteins are short-lived transcription factors that contain four highly conserved domains (referred to as domain I, II, III and IV). Aux/IAA proteins are thought to act as regulators of auxin-induced gene expression through heterodimerization of domains III and IV with ARF proteins thereby modifying ARF activity (Guilfoyle *et al.*, 1998a; Tiwari *et al.*, 2001; Tiwari *et al.*, 2004). In this model, auxin regulates transcription by stimulating the degradation of the Aux/IAA proteins through interaction of the Aux/IAA protein with the ubiquitin protein ligase SCF^{TIR1} complex (Gray *et al.*, 2001).

Given that in Arabidopsis 29 different Aux/IAA and 23 ARF proteins have been identified to date, the number of potential Aux/IAA – ARF interactions and the subsequent auxin responses, in the different tissues of a plant, are enormous.

Hence, elucidation of individual Aux/IAA gene functions has proven difficult since different mutant lines often exhibit unique as well as overlapping auxin related plant growth defects. Arabidopsis mutant screens have identified a number of Aux/IAA gain-of-function mutants where a mutation in domain II yields a more stable protein that is less sensitive to degradation (Rouse *et al.*, 1998; Worley *et al.*, 2000). These mutant lines confer dramatic auxin-related developmental defects, including altered gravitropism and apical dominance in *axr2/IAA7* (Nagpal *et al.*, 2000), and *axr3/IAA17* (Rouse *et al.*, 1998), lateral root defects in *IAA28* (Rogg *et al.*, 2001) and *IAA14* (Fukaki *et al.*, 2002), and photomorphogenic defects in *shy2/IAA3* (Tian *et al.*, 2002). In contrast, Overvoorde *et al.* (2005), analyzed a subset of insertion mutants, representing a loss-of-function mutation in Arabidopsis, but failed to show visible developmental defects in comparison to the wild type. These findings suggest a broad functional redundancy among the Aux/IAA gene family members making identification of biological function complex.

However, the analysis of Aux/IAA gene family members in other plant species may prove valuable when attempting to understand both common as well as process specific auxin dependent gene regulation. For example, the down regulation of a single Aux/IAA family member in tomato (*IAA9*) results in a dramatic pleiotropic phenotype, having simple leaves instead of wild type compound leaves and early fruit development, giving rise to parthenocarpy (Wang *et al.*, 2005).

Auxins have long been implicated to play a regulatory role in potato (*Solanum tuberosum*) tuber development. Endogenous auxin levels were found to be high just prior to and during stolon swelling after which auxin levels decreased (Koda and Okazawa, 1983). Furthermore, Xu *et al*. (1998), observed earlier tuberization when IAA was applied to single node cuttings in tuber-inducing medium. On the other hand, tuber formation was completely inhibited by high concentrations of IAA (Kumar and Wareing, 1974). In potato, only a single Aux/IAA (StIAA; Zanetti *et al.*, 2003) and ARF protein (ARF6; Faivre-Rampant *et al.*, 2004) have been described to date. *StIAA* expression levels increased after fungal infection, wounding or application of auxin (Zanetti *et al.*, 2003). *Arf6* expression levels are reduced in the apical meristem of the stolon tip at tuber onset and growth, and induced during meristem activation in dormant tuber buds (Faivre-Rampant *et al.*, 2004). The degree of auxin dependent gene regulation of developmental processes through the action of other Aux/IAA and ARF family members in potato plants is however still largely unknown. In this paper we describe the isolation and characterization of a novel potato Aux/IAA using a reverse genetics approach and discuss its potential functions in potato plant growth and development.

Results

Isolation of a potato Aux/ IAA gene family member (StIAA2)

In a previous study we analyzed gene expression changes during early potato tuber development (Kloosterman *et al.*, 2005). In this study, a number of genes were identified that showed a dramatic down-regulation in transcript levels during early tuberization. One of

these sequences showed strong homology to members of the Aux/IAA gene family. Determination of the sequence of potato clone (BI179192) provided a full length sequence with a predicted ORF of 213 amino acids and a molecular weight of 23.9 kDa (isoelectric point (pI); 9.14). Sequence comparisons with closely related Aux/IAA proteins from other species (*Arabidopsis thaliana* IAA14, IAA7, IAA17, IAA16; *Nicotiana tabacum,* IAA28 and poplar, *Populus tremula* x *Populus tremuloides* IAA6, IAA7) are shown in Figure 1 and clearly show the presence of conserved domains I-IV. Overall sequence homology of the predicted protein with the previously identified potato Aux/IAA protein (StIAA; 349 amino acids) reaches only 43%, with a significantly higher homology in the C-terminal end spanning domains III and IV (77%).

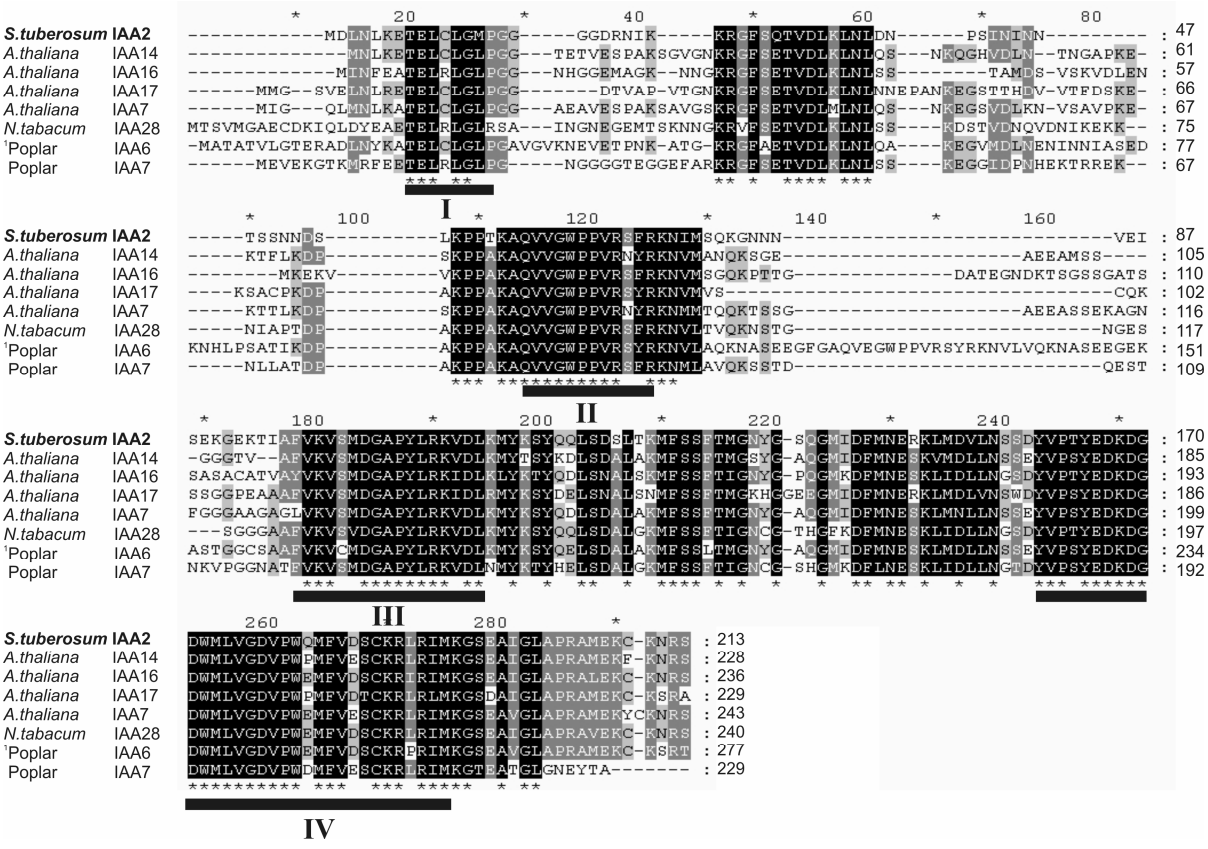

Figure 1; Sequence alignments of closely related Aux/IAA proteins reveals the presence of highly conserved domains indicated with black bars (I-IV). Proteins included in the alignment are: Solanum tuberosum StIAA2, Arabidopsis thaliana AtIAA14 (NP_193191), AtIAA17 (NP_171921), AtIAA16 (NP_187124), and AtIAA7 (NP_974355), Nicotiana tabacum Nt-IAA28 (AAD32146), [1]poplar (Populus tremula x Populus tremuloides) IAA6 (CAC84710), and IAA7 (CAC84711).

Based on its sequence homology with the identified Aux/IAA proteins, the predicted protein was classified as a novel potato Aux/IAA family protein and was named StIAA2.

In order to assess the expression profile of *StIAA2* in potato plants, quantitative RT-PCR was performed on a set of different potato tissues (Figure 2). Transcript levels were found to be highest in the petiole, non-tuberizing stolon tip (NTS), shoot apex, leaf and stem.

Lower levels of expression were detected in root, tuber and stolon. Very low expression of *StIAA2* was detected in dormant tubers stored at 4˚C.

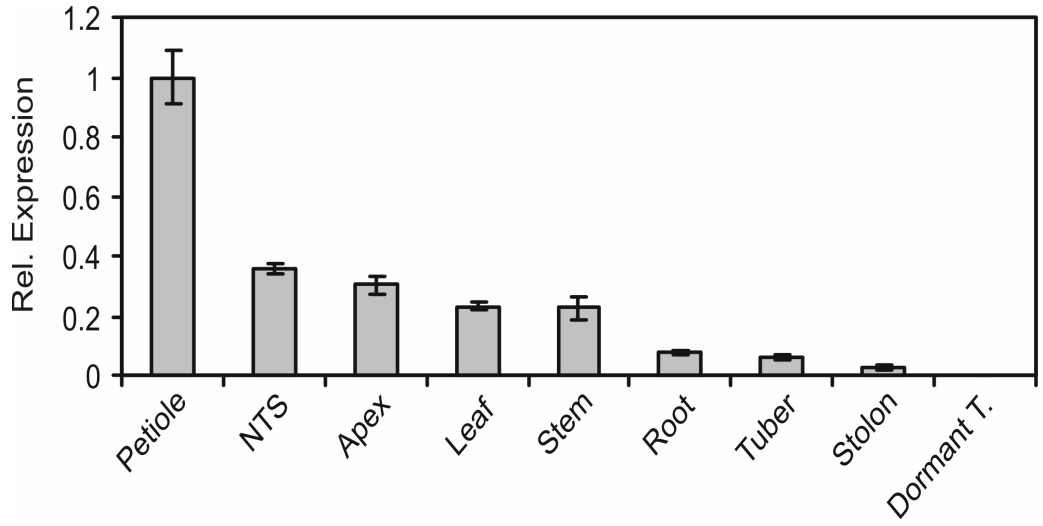

Figure 2; Comparison of the relative expression levels of *StIAA2* in different tissues using qRT-PCR. Expression levels were calculated relative to the tissues in which expression level was highest (Petiole). Tissues studied include, petiole, non-tuberizing stolon tip (NTS), shoot apex, leaf, stem, root, tuber, stolon and dormant tuber (stored for 4 weeks at 4˚C).

Differential expression during potato tuber development

In a previous study, using microarray technology, we found a potato EST representing *StIAA2* that showed strong down-regulation during potato tuber development (Kloosterman *et al.*, 2005). A down-regulated expression profile of *StIAA2* during potato tuber development was confirmed using quantitative RT-PCR. A subset of the different developmental stages that were studied are represented in Figure 3A and include; non-tuberizing stolon tip grown under long day conditions (16h), non-tuberizing stolon under tuber inducing short day conditions (8h), tuber initiation and subsequent tuber growth stages. Expression levels were calculated and plotted relative to an internal standard and the first harvest time point (ΔΔCt). After the first harvest under LD conditions, plants were transferred to SD conditions (t = 0 days) and subsequent harvest stages are represented on the x-axis as days after the switch from LD to SD. A sharp decline of *StIAA2* transcript levels was observed at the time point of tuber organogenesis (Figure 3B; *StIAA2* stage 3 and 4; ΔΔCt -2.4 and ΔΔCt -3.1, respectively) indicating strict transcriptional control during these stages. To check for a common expression pattern of Aux/IAA gene family members, gene specific primers were designed for the only other potato Aux/IAA gene characterized, *StIAA*. Transcript levels of *StIAA* at tuber organogenesis (Figure 3B; *StIAA* stages 3 and 4) exhibit a small increase (2-fold) after which gene expression returns to basal levels. The expression patterns of *StIAA* and *StIAA2* have an opposite pattern of expression during tuber organogenesis. However, *StIAA2* shows a much larger difference in expression levels, indicating different mechanisms of transcriptional control or tissue specificity for both Aux/IAA family members during early potato tuber development.

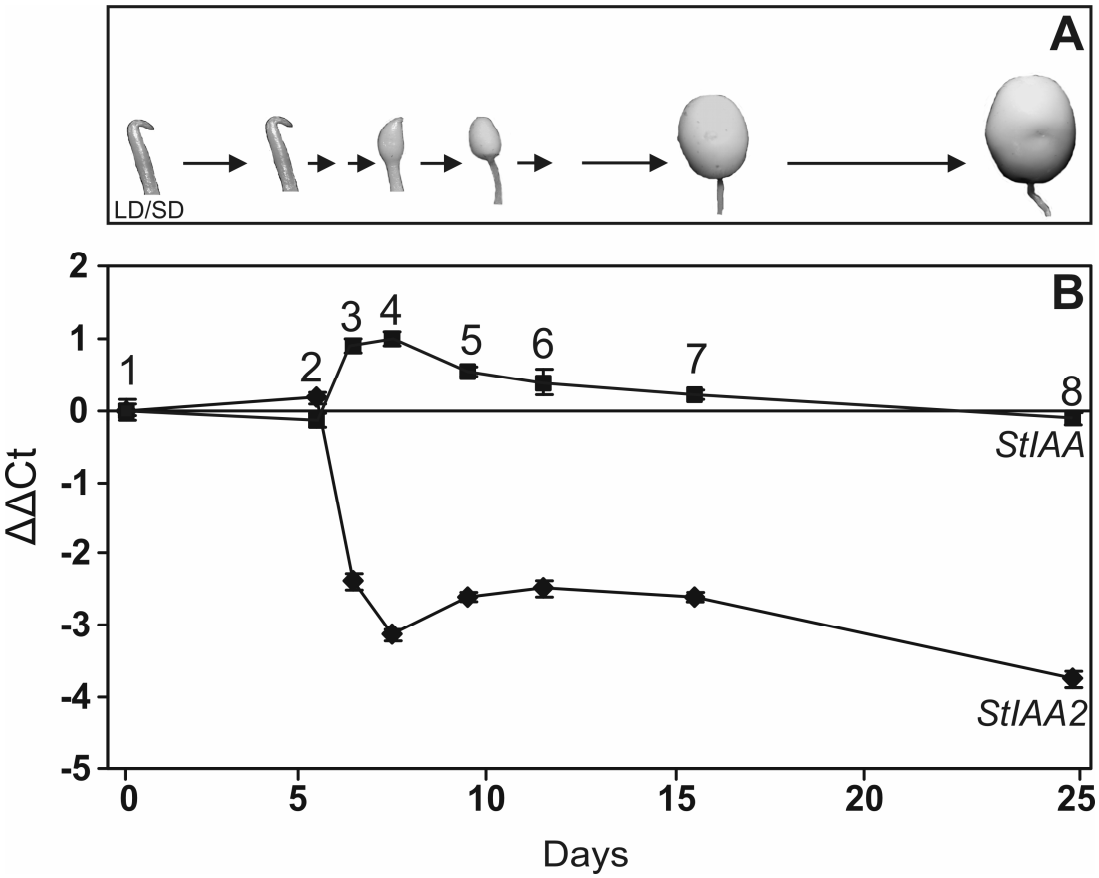

Figure 3; Quantitative RT-PCR measuring gene expression of two potato Aux/IAA proteins, StIAA and StIAA2 during potato tuber development. RNA derived from eight tuber developmental stages (1-8) harvested over a period of 25 days after a switch from 16h to a 8h light period (t=0). (A) Upper panel shows a subset of the different growth stages that can be found during development. (B) Lower panel shows the expression levels of *StIAA* and *StIAA2* genes relative to internal standard and the first harvest time point (ΔΔCt). Error bars indicate the standard deviation between independent measurements.

Suppression lines of StIAA2 exhibit several altered growth responses

Transgenic potato plants were produced containing either the *StIAA2* over-expression or suppression construct. Twenty-seven transgenic clones harbouring the suppression construct and fourteen over-expression clones were transferred to the greenhouse. Plants were grown in triplicate and scored for altered growth characteristics. For the over-expression clones, no visible differences in growth patterns were observed in the aerial parts of the plant and underground tuber formation in comparison to the untransformed control plants (data not shown). In sharp contrast, over 30% of the suppression clones did show several dramatic phenotypes. Plants were significantly taller in comparison to untransformed control plants and exhibited a lower petiole angle relative to the main stem resulting in petioles that grow in a more vertical manner (hyponasty) (Figure 4a,f and Table I). The petiole angles are lowest around the upper nodes and comparable to control plants in the basal regions. Furthermore, the shoot apex showed exaggerated curvature of growing leaf primordia (Figure 4b-d) and similar deformations were observed in the apices of axillary buds and lateral shoots.

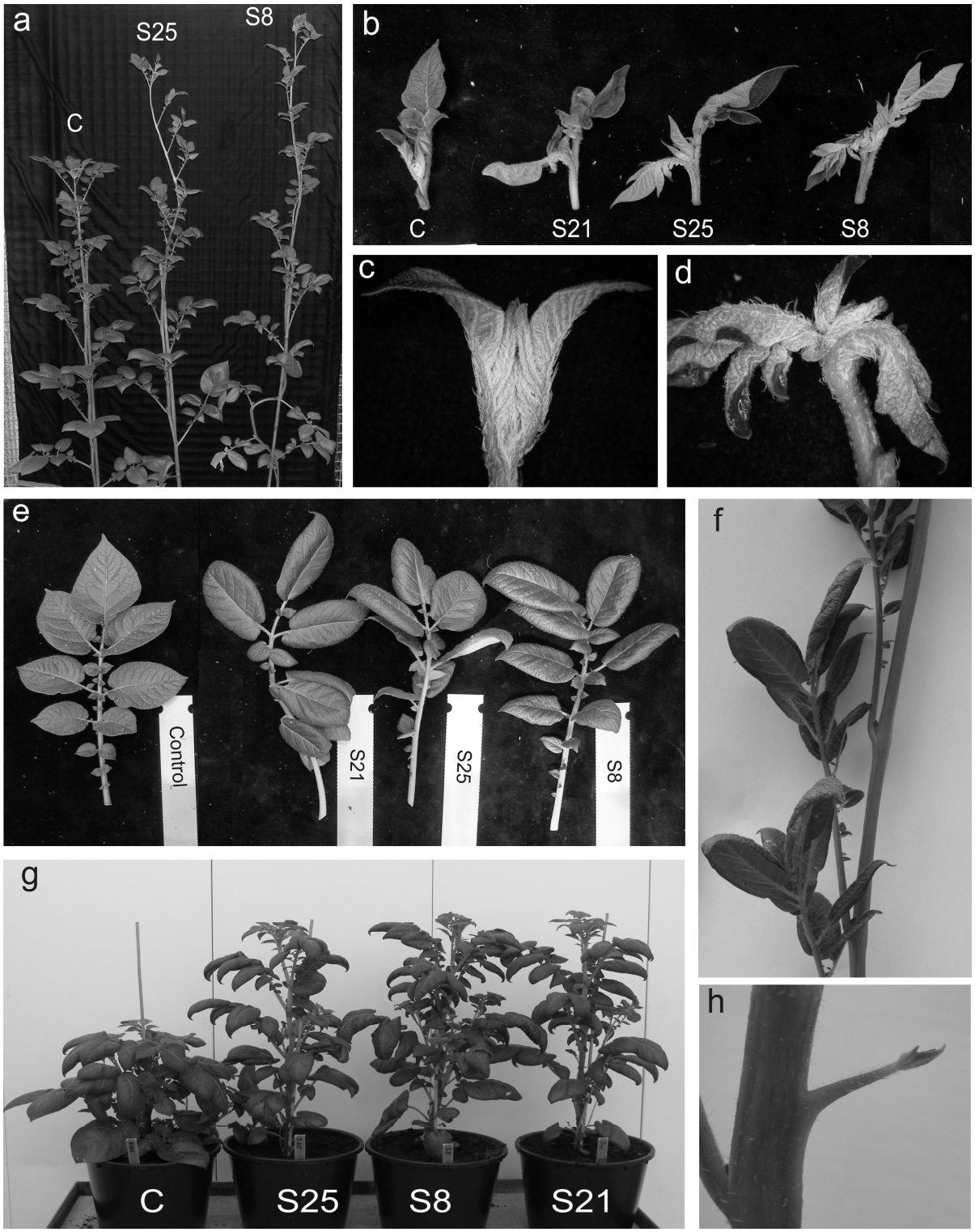

Figure 4; Growth phenotypes of *StIAA2* down regulated plants grown in the greenhouse (a-f) or in a growth cabinet (g,h). Increased plant height of 10-week old plants of *StIAA2* suppression clones (S8, S25) compared to untransformed control plants (a). Exaggerated curvature of developing leaf primordia around the shoot apex of S8, S21 and S25 in comparison to control (b). Photograph of shoot apex of control (c) and suppression clone S21 (d). Downward curling and twisted leaf positioning of leaves in the *StIAA2* suppression clones (e). Hyponastic growth of young petioles and twisted leaf positioning (f). Comparison of plant height in controlled climate conditions of StIAA2 suppression clones (S8, S21 and S25) and untransformed control (g). Aborted leaf primordia initiating from the primary stem in StIAA2 suppression clone S21 (h).

Table I: Average[1] plant height, internode length and petiole angle of *StIAA2* suppression clones and untransformed control plants

	Avg. Plant Height (cm)	Avg. Internode length (cm)	Avg. Petiole angle of third and 6th node (degrees)
Control	**119 ± 10.4**	**7.0 ± 0.4**	**32.7 ± 5.7**
*StIAA2*_S6	158 ± 16.4	8.1 ± 0.6	21.3 ± 3.8
*StIAA2*_S8	161 ± 12.7	8.5 ± 0.5	21.8 ± 3.6
*StIAA2*_S11	160 ± 20.5	8.2 ± 0.7	15.8 ± 5.7
*StIAA2*_S22	156 ± 14.8	7.6 ± 0.5	15.5 ± 5.5
*StIAA2*_S25	162 ± 8.1	8.3 ± 0.3	21.0 ± 5.7

[1]Averaged values and standard deviations were calculated from three independent measurements.

This curvature in young developing leaf primordia most likely has an effect on further leaf development and could partially explain the observed hyponastic growth pattern of the petiole and subsequent twisted leaf positioning in fully expanded leaves (Figure 4e).

In addition, fully expanded leaves were curled downwards. Another interesting phenotype that, however, occurs at low frequency in a subset of transgenic clones, is the presence of aborted leaf primordia (Figure 4h). Surprisingly, no clear difference in the transgenic plants could be observed with regard to growth characteristics of underground stolons and tuber formation. Averaged stolon length and tuber yield was comparable to the untransformed control (data not shown). For expression studies, the three clones showing the strongest overall phenotypes were selected and checked for the presence of the suppression construct. Plants were grown in eight repeats under controlled conditions in a growth cabinet (16h light at 20˚C/18˚C) to allow a more detailed comparison between the transgenic and untransformed control plants.

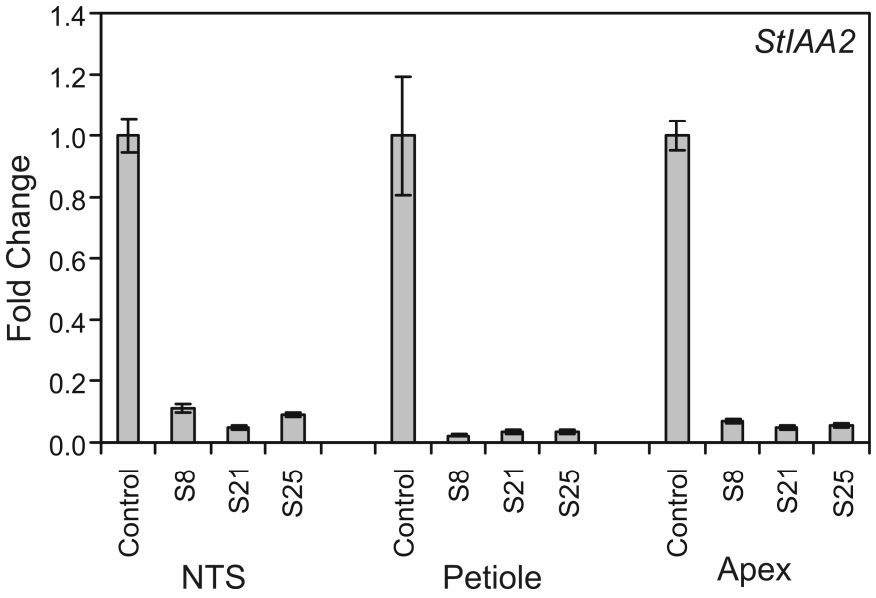

Figure 5; Quantitative RT-PCR of *StIAA2* expression levels in the suppression clones (S8, S21 and S25) in different tissues (NTS, petiole and shoot apex) relative to the untransformed control plants. Error bars indicate the standard deviation between three independent measurements.

Phenotypes observed under the controlled climate conditions were similar to the ones found in the greenhouse experiment, although overall plant height was lower and differences in petiole angle less dramatic (Figure 4g). Different plant tissues (shoot apex, petiole, NTS) were harvested in replicates to determine the level of *StIAA2* gene suppression. Transcript levels were measured using qRT-PCR and were calculated relative to the untransformed control plants. In all silencing clones and tissues, *StIAA2* transcript levels were significantly reduced up to 10-fold as shown in Figure 5.

Downstream effects of reduced StIAA2 expression

Since Aux/IAA proteins are important regulators of auxin mediated responses and some of the phenotypes found in *StIAA2* suppression plants can be identified as enhanced auxin responses, we have screened these clones for altered gene expression using a dedicated potato cDNA-microarray. Based on the strong phenotypes observed in the suppression clones and the high endogenous expression levels of *StIAA2* in the shoot apex, this tissue was selected for transcript profiling. Labelled samples of *StIAA2* transgenic clones S21 and S25 were hybridized in repeats including a swap dye. After data normalization and statistical analysis, genes that are significantly differentially expressed (>2fold) in both transgenic clones, are listed in Table II. Interestingly, an EST with high homology to *StIAA*, the only other identified Aux/IAA family member in potato, is down-regulated 2.4- and 4.0-fold in transgenic clones S25 and S21 respectively. To confirm that *StIAA* is down-regulated in both suppression clones, qRT-PCR with *StIAA* gene specific primers was performed on isolated material form the shoot apex and the additional tissues, NTS and petiole. Lowered *StIAA* transcript levels were confirmed in all tissues analyzed (Figure 6).

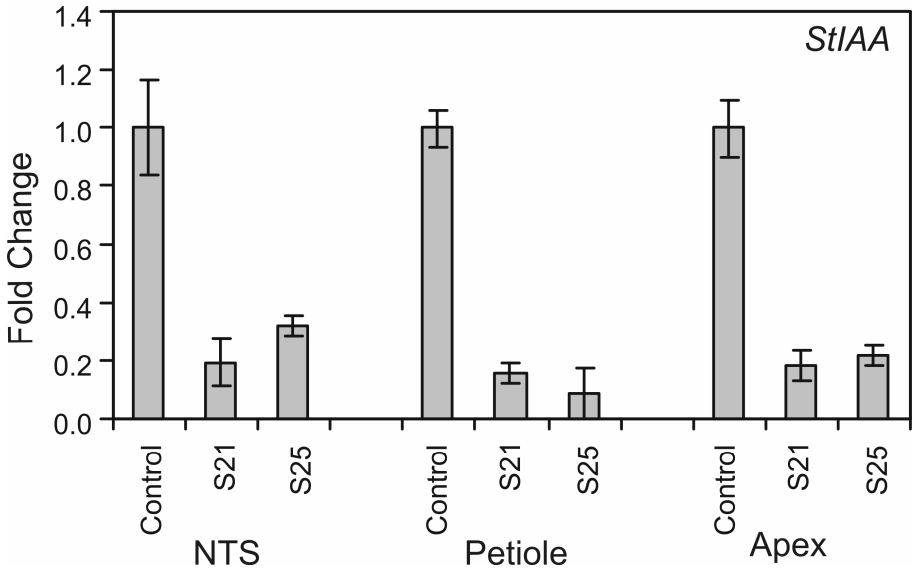

Figure 6; Confirmation of lowered StIAA expression in StIAA2 suppression clones (S21 and S25) in harvested tissues (NTS, petiole and shoot apex) relative to the untransformed control plants. Error bars indicate the standard deviation between three independent measurements.

Table II: Differential gene expression in the shoot apex of *StIAA2* suppression clones (S21, S25) in comparison to untransformed control plants

Clone Access. nr	Gene homology	Fold Down regulation	
		S25	**S21**
BI178011	StIAA [*S. tuberosum*]	-2.4	-4.0
BG098056	DWARF1/DIMINUTO [*S. lycopersicum*] (Brassinosteroid biosynthetic protein)	-3.8	-5.6
BG596458	DWARF1/DIMINUTO [*S. lycopersicum*] (Brassinosteroid biosynthetic protein)	-4.1	-5.1
BE340263	Cellulose synthase catalytic subunit [*A. thaliana*]	-4.2	-4.1
BG095782	Cytochrome P450 [*S. lycopersicum*]	-2.4	-3.2
BG594226	Cytochrome P450 [*S. tuberosum*]	-2.7	-2.9
BE923430	Tyrosine/dopa decarboxylase [*P. somniferum*]	-3.1	-2.7
BE344117	Arginine decarboxylase 1 [*D. stramonium*]	-2.8	-3.0
BG600942	Arginine decarboxylase 1 [*D. stramonium*]	-2.4	-3.3
BG593608	Phenylalanine ammonia-lyase [*S. lycopersicum*]	-2.4	-2.0
BE344057	Phenylalanine ammonia-lyase 1 [*S. tuberosum*]	-2.1	-2.9
BG593871	Ent-kaurenoic acid oxidase [*H. vulgare*]	-2.0	-2.9
BI175804	Ent-kaurenoic acid oxidase [*C. maxima*]	-3.6	-2.9
BF188276	2-oxoglutarate-dependent dioxygenase [*S. tuberosum*]	-4.3	-4.6
BG596984	2-oxoglutarate-dependent dioxygenase [*S. chacoense*]	-4.2	-5.5
BG598323	UDP-glucose glucosyltransferase [*S. tuberosum*]	-4.3	-8.3
BE340276	UDP-glucose glucosyltransferase [*S. tuberosum*]	-4.7	-3.9
BI179665	Aspartic protease inhibitor 8 precursor [*S. tuberosum*]	-2.5	-2.3
BI179515	Major intrinsic protein 2 [*S. tuberosum*]	-2.5	-4.0

Within the shoot apex, *StIAA* expression was down-regulated 4.6-fold and 5.6-fold for clones S25 and S21 respectively, indicating an underestimation of differential expression in the microarray data set based on the higher sensitivity of qRT-PCR technology. The finding of reduced transcript levels for *StIAA* in the shoot apex of *StIAA2* transgenic clones was unexpected since contrasting expression profiles were observed for *StIAA* and *StIAA2* during potato tuber development (Figure 3), indicating independent transcriptional regulation at least during tuber formation. Furthermore, two ESTs showed high homology to a brassinosteroid biosynthetic protein (DWARF1/DIMINUTO), which is intriguing since Aux/IAA proteins have been suggested to play a role in brassinosteroid sensitivity and signalling. Other genes

identified as being differentially expressed in the *StIAA2* suppression clones have a wide variety of functions often represented by multiple independent clones strengthening their significance. For example, two different clones with homology to UDP-glucose glycosyl transferases are severely down-regulated in both transgenic clones. Similarly, two 2-oxo-glutarate dependent-dioxygenases, two arginine decarboxylases, two ent-kaurenoic acid oxidases, and two different clones representing phenylalanine ammonia-lyase (PAL) were identified (Table II). Surprisingly, no genes were detected in the suppression clones that exhibited a significant increase in transcript levels, suggesting that StIAA2 has primarily a promoting effect on auxin responsive gene expression.

Discussion

Based on the high sequence homology with other Aux/IAA family members and the presence of all four highly conserved domains, we have isolated a potato Aux/IAA gene that was named *StIAA2*. Plants with reduced *StIAA2* expression exhibit a number of dramatic growth alterations in the aerial parts of the plant including, increased plant height, petiole hyponasty and curvature of leaf primordia in the shoot apex. The observed curling of leaves and differences in plant height are common characteristics of differential auxin responses identified in Arabidopsis Aux/IAA mutant lines (reviewed in Liscum and Reed, 2002) and in tomato (Wang *et al.*, 2005), suggesting a conserved role for Aux/IAA proteins in the regulation of these responses in plants. A more unique phenotype that was observed in our *StIAA2* suppression clones in relation to Aux/IAA protein functional studies, is the hyponastic growth pattern of young petioles. In an early study by Kazemi *et al.* (1974), it was shown that decapitation of the main shoot of tomato induces hyponasty of young leaves and the application of auxin to the cut apex produces an epinastic growth response associated with ethylene production. Similarly, Cox *et al.* (2004), have shown that the hyponastic growth response leading to the upward movement and growth of young petioles in submerged *Rumex palustris* is a result of differential cell elongation across the petiole base. Within this response, differential auxin distribution is likely to play a role in the maintenance of the hyponastic growth pattern in which ethylene, ABA and gibberellin play regulatory and inducing roles. The observation that the hyponastic growth pattern in the suppression clones is highest in the upper nodes and lower in the basal part of the plant is consistent with a normal auxin distribution pattern. In this respect the shoot apex, the primary site of auxin synthesis, exhibits one of the most severe growth defects, the curvature of developing leaf primordia in the shoot apex (Figure 4b,d). Whether the hyponastic growth pattern of young petioles is a continuation of the observed curvature of leaf primordia in the shoot apex remains to be investigated. Taken together, StIAA2 may play a role in the auxin-mediated growth and development of young petioles, supported by its high expression within these tissues. It is presently unclear whether the observed phenotypes in the suppression clones are a result of differential cell elongation in specific cell types or regions within the tissues studied. In order to ascribe the observed downward curling of leaves in the transgenic plants to an increase in average cell size, we measured epidermal cell sizes on the adaxial surface of

control and transgenic plants, however, no significant differences could be found (data not shown).

A number of Aux/IAA proteins have been shown to function as transcriptional repressors through interaction with ARF proteins that have the capacity to induce or suppress the transcription of auxin responsive genes (Guilfoyle *et al.*, 1998a; Guilfoyle *et al.*, 1998b; Tiwari *et al.*, 2001). Studying gene expression in the shoot apex of two suppression clones in comparison to the untransformed control using a dedicated cDNA-microarray, we detected significant down regulation of 19 different ESTs with often overlapping functional homologies (Table II). Although the dedicated potato array used in our studies is enriched for hormone regulated genes, it contains a relative small number of genes (<2000) (Kloosterman *et al.*, 2005). Therefore, it is difficult to assess the importance of the identified genes in producing the observed phenotypes, since the total number of genes that are being either down-regulated or up-regulated in the *StIAA2* silencing clones, is expected to increase significantly when based on the entire transcriptome.

In our experiment we found that one of our down regulated ESTs shows strong homology to another Aux/IAA family member in potato, StIAA. Gene specific qRT-PCR confirmed the down regulation of *StIAA* in the shoot apex as well as in the other tissues studied (Figure 6). Differential gene expression of other family members in Aux/IAA down regulated plants has been described for tomato, where down regulation of *IAA9* results in increased transcript levels of *IAA3* (Wang *et al.*, 2005). In this system IAA9 acts as a transcriptional repressor of *IAA3*. In potato plants therefore, one could hypothesize a role for StIAA2 as transcriptional inducer of *StIAA* expression. However, during the early stages of potato tuber development, expression of *StIAA2* is rapidly decreasing whilst *StIAA* expression exhibits a small transient increase. This apparent contradiction may be explained if both genes were to be expressed in different types of tissue within the stolon and growing tuber. Another explanation may be that the transcriptional control of *StIAA* is also dependent on the presence of other Aux/IAA and ARF proteins that are absent or lowly expressed in the developing tuber. *StIAA2* and *StIAA* do not share more than 61% homology at the nucleotide level and therefore cross-silencing of *StIAA* through the *StIAA2* suppression construct seems unlikely but can not be ruled out completely.

Brassinosteroids (BR's) are plant hormones that have important functions in plant growth and development very similar to Auxins. Auxins and BR's are known to act both independently as well as synergistically regulating transcription of a unique and similar subset of genes (Goda *et al.*, 2002; Nemhauser *et al.*, 2004). Furthermore, Aux/IAA proteins function in BR signalling pathways modulating BR sensitivity in a manner dependent on organ type (Nakamura *et al.*, 2006). Interestingly, in our experiment we found that gene expression of a brassinosteroid biosynthetic protein (DWARF1/DIMINUTO; Klahre *et al.*, 1998) was reduced in the *StIAA2* suppression clones. The differential expression of a BR biosynthesis gene further supports the existence of crosstalk between Aux/IAA and brassinosteroid signalling and regulation as described in a number of research papers (Goda *et al.*, 2004; Halliday, 2004; Nemhauser *et al.*, 2004; Nakamura *et al.*, 2006). Other down regulated genes that were detected in the shoot apex of both suppression clones have a wide

variety of known and undefined plant functions that may or may not contribute to the observed phenotypes. Whether these genes are down regulated directly as a result of *StIAA2* gene suppression or indirectly by the altered plant stature or metabolic status still remains to be determined.

Auxin is expected to play an important role in potato tuber development (Kumar and Wareing, 1974; Koda and Okazawa, 1983). Therefore, *StIAA2* was initially regarded as a good candidate for playing a role in auxin mediated stolon and tuber development, based on its high expression in non-tuberizing stolon and its strong down-regulated expression profile during tuber organogenesis (Figure 3). However, we were not able to detect a visible growth defect in any of the underground organs, suggesting StIAA2 may exert its function(s) more strongly in light grown tissues. In addition, a much suggested explanation for the finding of no visible phenotypes in Aux/IAA mutant lines is the presence of overlapping functions of other Aux/IAA family members within specific plant tissues (Liscum and Reed, 2002; Overvoorde *et al.*, 2005). The lack of visible phenotypes has become a recurring problem in Arabidopsis Aux/IAA loss-of-function mutants as clearly shown in a recent study by Overvoorde *et al.* (2005). Therefore, it was surprising to find such distinctive phenotypes in the transgenic potato clones with reduced *StIAA2* expression resembling a 'loss of function' mutation. Similarly, in tomato the down regulation of *IAA9* results in dramatic alterations in leaf morphology and fruit development indicating a specific and unique role for IAA9 in this process (Wang *et al.*, 2005). These findings underline the importance of analyzing different AUX/IAA proteins in a number of different plant species. Furthermore, analysis of Aux/IAA protein functions in other plant species with unique developmental processes (for example tuber formation), enhances the possibility of discovering novel pleiotropic growth defects mediated by members of the Aux/IAA gene family.

Experimental procedures

Cloning of StIAA2 and sequence analysis

The full length sequence of the potato *StIAA2* was obtained by sequencing cDNA clone BI179192 deriving from an *in vitro* grown microtuber EST library (cv. Bintje) using vector based primers T3 and T7. Homology searches were carried out using the blastX program (Altschul *et al.*, 1990). Protein alignments of closely related Aux/IAA genes identified in other plant species were carried out using the ClustalX program (Thompson *et al.*, 1997).

Plant Transformation

The complete coding sequence of *StIAA2* was amplified by PCR (forward primer 5'-CACCATGGACTTGAATCTCAAGGA-3' and reverse primer 5'- TGCTTTCATTCTTCATCAAC-3'). Forward primer contains a 5'-CACC partial overhang to facilitate cloning of the PCR product in pENTR/SD/D-TOPO vector (Invitrogen, the Netherlands). *StIAA2* was further subcloned

by a LR recombination reaction into Gateway™ plant transformation destination vector pk7WG2 and pk7GWIWG2 (Plant Systems Biology, University of Ghent, Belgium) (Karimi *et al.*, 2002). The two resulting expression constructs were named pk7*StIAA2*_O and pk7*StIAA2*_S. pk7*StIAA2*_O expression results in heterologous expression of *StIAA2* under control of the 35S-CaMV promoter. The pk7*StIAA2*_S expression construct consists of a full length *StIAA2* inverted repeat harbouring an intron behind the 35S-*CaMV* promoter that when expressed produces a hairpin structure resulting in post transcriptional gene silencing (PTGS). pk7*StIAA2*_O and pk7*StIAA*_S were transformed into *Agrobacterium tumefaciens* strain *Agl0* using electroporation. *In vitro* shoots of the *Solanum tuberosum* cv. Karnico were used for *A. tumefaciens* mediated transformation (Visser *et al.*, 1991). After regeneration of *in vitro* shoots on selective kanamycin MS medium (100 mg/L) (Murashige and Skoog, 1962), the shoots were transferred to the greenhouse to produce mature plants. Plants regenerated from untransformed *in vitro* shoots were used as wild type controls.

Plant material and measurements

Regenerated transformed plants were grown in 5L soil-filled pots in the greenhouse and regularly scored for growth alterations in comparison to control plants. Due to low light conditions within the greenhouse, plants were provided with additional lighting through high pressure sodium lamps (Philips, the Netherlands) to maintain a 16h light period. From a subset of plants exhibiting increased growth and the control plants, plant height was measured as the distance from the shoot apex to the pot rim and average plant height and internode length was calculated from three independent replicates after twelve weeks of plant growth. From the same set of plants average petiole angles were calculated by measuring the angle between the petiole base and main stem axis from the third and sixth node counted from the first node having a fully expanded leaf. The three suppression clones exhibiting the strongest overall phenotypes were selected for more detailed analysis and gene expression studies. Integration of at least one copy of either construct in the selected transgenic plants was confirmed by PCR using 35S-*CaMV* promoter sequence specific primers (forward primer 5'-GCACCTACAAATGCCATCA-3' and reverse primer 5'-GATAGTGGGATTGTGCGTCA-3'). Six replicates of *in vitro* untransformed control plants and of the three selected suppression clones were transferred to soil and grown in a climate chamber under controlled conditions (16 h light 20˚C and 8h dark at 18˚C). After 6 weeks of plant growth, tissues including, leaf, petiole, shoot apex were harvested for two independent replicates of the three suppression clones and untransformed control plant. The collected material was immediately frozen in liquid nitrogen and stored at -80˚C till further analysis.

RNA isolation and quantitative RT-PCR

For expression studies within the different transgenic clones and control plants, total RNA (tRNA) was isolated as described by Bachem *et al*. (1996). For expression studies of the *StIAA2* and *StIAA* genes during the potato tuber developmental time series and potato

tissues, tRNA was used from a previous study (Kloosterman *et al.*, 2005). One additional tissue sample was included, namely the shoot apex, and was harvested from 8 week old greenhouse grown plants (*S. tuberosum* cv. Karnico). Relative expression levels of genes were determined by qRT-PCR on a Perkin Elmer Abi Prism 7700 Sequence detector (Perkin Elmer, Niewerkerk, the Netherlands) following the protocol described in 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: *StIAA2* forward primer 5'-TGATTCATGCAAGCGTTTACG-3', reverse primer 5'-TGCAAGTCCAATGGCTTCTG-3'; *StIAA* (AY098938) forward primer 5'-CCTTGGGAGATGTTTATTGACAC-3', reverse primer 5'-TCCGACACTTTTCCATAGCC-3' and *ubi3* (L22576) forward primer 5'- TTCCGACACCATCGACAATGT-3', reverse primer 5'-CGACCATCCTCAAGCTGCTT-3'.

Microarray hybridization and data analysis

mRNA was purified from isolated tRNA from the shoot apex of suppression clones S21, S25 and untransformed control plants using the GenElute™ mRNA miniprep kit (Sigma Aldrich, Zwijnberg, the Netherlands). First strand cDNA synthesis followed by target labelling was performed using the SuperScript™ Indirect cDNA Labelling System (Invitrogen, the Netherlands), according to the manufactures protocol. Cy3 or Cy5 labelled targets from both suppression clones were hybridized independently against the labelled untransformed control target. A swap dye experiment was included for both suppression clones. Potato tuber life cycle cDNA microarray slides were pre-hybridized and processed as described in (Kloosterman *et al.*, 2005). Hybridization of the target samples was performed in the HybArray12™ (Perkin Elmer, Niewerkerk, the Netherlands) hybridization station at 42˚C over a period of 20 hours. Following hybridization, slides were washed as described in van Doorn *et al.* (2003). Slides were immediately scanned using a Scanarray®ExpressHT scanner according to the manufactures specifications (Perkin Elmer, Niewerkerk, the Netherlands). Spotfinding, data extraction, LOWESS normalization and several quality control filters (Spot quality, Low intensity threshold, Signal to Noise Ratio) were performed with the ScanArray® (Perkin Elmer, Niewerkerk, the Netherlands) and the Microsoft® Excel software packages. Probes on the potato cDNA microarray were spotted in triplicate and were analyzed separately to produce a potential number of 6 independent measurements for each suppression clone. Detection of significantly differentially expressed genes shared between both analyzed suppression clones was performed using the SAM software (Tusher *et al.*, 2001) with a set FDR of 0. The identified differentially expressed gene set was imported into the SPSS software package and were subjected to further T-testing ($p<0.005$) for significant expression change >2-fold in both directions.

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Chapter 5

A differentially expressed potato cell wall protein is identified as a candidate gene important for determining tuber cooking type using a bulk segregant analysis approach

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Abstract

The implementation of functional genomic tools in plant QTL analysis is likely to greatly enhance candidate gene identification and subsequent cloning steps by treating variability in the expression of genes as quantitative phenotypes. In this chapter we have studied gene expression with a dedicated cDNA microarray in potato (*Solanum tuberosum*) tubers using a bulk segregant analysis approach leading to the identification of a novel candidate gene involved in determining potato tuber texture after cooking. The candidate gene shows strong homology to the tyrosine and lysine rich protein family and was named *StTLRP*. Differences in gene expression of the candidate gene were confirmed by quantitative RT-PCR for 56 genotypes from a segregating diploid potato population. The identified potato *StTLRP* gene was mapped both as an expression QTL and PCR marker to the same chromosomal location as the QTL identified for potato tuber texture after cooking. By implementing a pooling strategy for expression analysis, we have successfully identified a candidate gene (*StTLRP*) involved in determining tuber cooking characteristics substantiating the potential of genetical genomics.

Introduction

Differential gene expression within a population can be considered as a quantitative trait that can result in the mapping of gene expression as a proper QTL or so-called eQTL (Schadt *et al.*, 2003). The combination of expression profiling and genetics has been referred to as 'genetical genomics' and is expected to greatly advance our capabilities to resolve metabolic, regulatory and developmental pathways (Jansen and Nap, 2001). The use of microarray technology for accurately scoring of differential gene expression within large populations has already resulted in the identification of novel candidate genes underlying specific traits of interest (Brem *et al.*, 2002; Wayne and McIntyre, 2002; Schadt *et al.*, 2003; Kirst *et al.*, 2004). Efficient analysis of the entire transcriptome is however still limited to organisms of which the genome sequence has been largely determined or for which comprehensive EST databases are available.

In a previous study (Kloosterman *et al.*, 2005), we have developed a dedicated cDNA-microarray, containing around 2000 elements specifically designed for studying gene expression during potato tuber development and tuber quality traits. In the study described here, we implement a novel pooling strategy, or bulk segregant analysis approach (BSA; Michelmore *et al.*, 1991), for studying gene expression in a diploid potato population in order to identify novel candidate genes specifically involved in the determination of the textural changes in potato tubers after steam cooking (i.e. cooking type).

Texture of cooked potatoes is an economically important quality aspect and is generally characterized as the differences between mealy and non-mealy/firm tubers. A mealy tuber is one which, while it retains its form on cooking, may readily be broken down with a fork to give a dry crumbly mash through separation of individual cells (Burton, 1966). A firm tuber on the other hand, does not break down easily in comparison to mealy tubers but

when forced is accompanied by a significant amount of cell breakage (Burton, 1966; van Marle *et al.*, 1992). Textural changes occurring during cooking are mainly associated with cell wall and middle lamella structural components and the gelatinization characteristics of starch (van Marle, 1997; Alvarez and Canet, 1998). One of the parameters to categorize cooking behaviour is sloughing: the loosening of the outer layers of the cooked potato. Sloughing is determined by the amount of intercellular adhesion within cooked potato tissue (Jarvis and Duncan, 1992; van Marle *et al.*, 1992). Van Marle *et al*. (1994), showed that both cell sloughing and the release of pectic materials were higher for the mealy cooking potato cultivar Irene than for the non-mealy cooking cultivar Nicola. Furthermore, a difference in cell wall thickness was observed, accompanied by a difference in the degree of middle lamella breakdown during cooking which was higher in cultivar Irene in comparison to cultivar Nicola (van Marle *et al.*, 1997). Studies on the assembly process of cell walls have provided insight in the architecture of the primary cell wall and led to the identification of a number of genes and structural proteins that are associated with modifying cell wall characteristics such as permeability, expansion, stress relaxation and mechanical strength (Showalter, 1993; Cosgrove, 1997). However, the genetic components and genes involved in potato tuber cell wall characteristics in relation to the differences in cooking type have not been fully understood and there is clear lack of high-quality candidate genes.

In this paper we combine the power of integrating phenotypic data with quantitative analysis of gene expression and genetic map information for the discovery of candidate genes determining potato tuber cooking type. By using this approach we were able to identify a differentially expressed cell wall gene in bulks of genotypes representing contrasting tuber cooking types and demonstrate the power of pooling strategy in finding candidate genes based on expression differences.

Results

Distribution of potato tuber texture after cooking scores

The offspring of a diploid backcross between diploid parents C and E exhibits strong segregation for a large number of tuber quality traits including potato tuber cooking type. Textural changes of tubers after cooking (i.e. cooking type) from individuals of the population were determined in two consecutive years. Texture of steam cooked potato tubers was visually scored and categorized on a nominal scale ranging from firm/non-mealy (1) to extreme mealy tubers (6). Figure 1 shows an example of the differences that could be observed in the texture of potato tubers within the CxE population after cooking, including a mealy (Figure 1A) and a firm/non-mealy tuber genotype (Figure 1C). The outer cell layers appear to be shed off (sloughing) and the loose layers can be typified as slurry consisting of intact individual cells as shown in the enlargement of part of the tuber surface (Figure 1B). Non-mealy tubers maintain a compact appearance having a relatively firm and glossy surface (Figure 1D). Cross-sections of these tubers reveal similar phenotypes in the inner parts of the tuber although in general less severe as observed in the outer layers. Potato tuber cooking

type as a quantitative trait shows a high level of heritability (h^2) in both harvest years (1998; 0.95 and 1999; 0.80), indicating the observed variance in both years is primarily due to genotypic variation present within the population. In addition, a strong correlation (0.85) was found between the individual datasets of the two harvest years and average texture scores were calculated for 226 genotypes by combining the two datasets. The distribution of the number of plants over the different texture classes is shown in Figure 2. Potato tuber cooking type within the CxE population shows a transgressive segregation in which both parental clones exhibit a relatively firm/non-mealy texture profile (1.0 and 1.8 for the C and E parent, respectively).

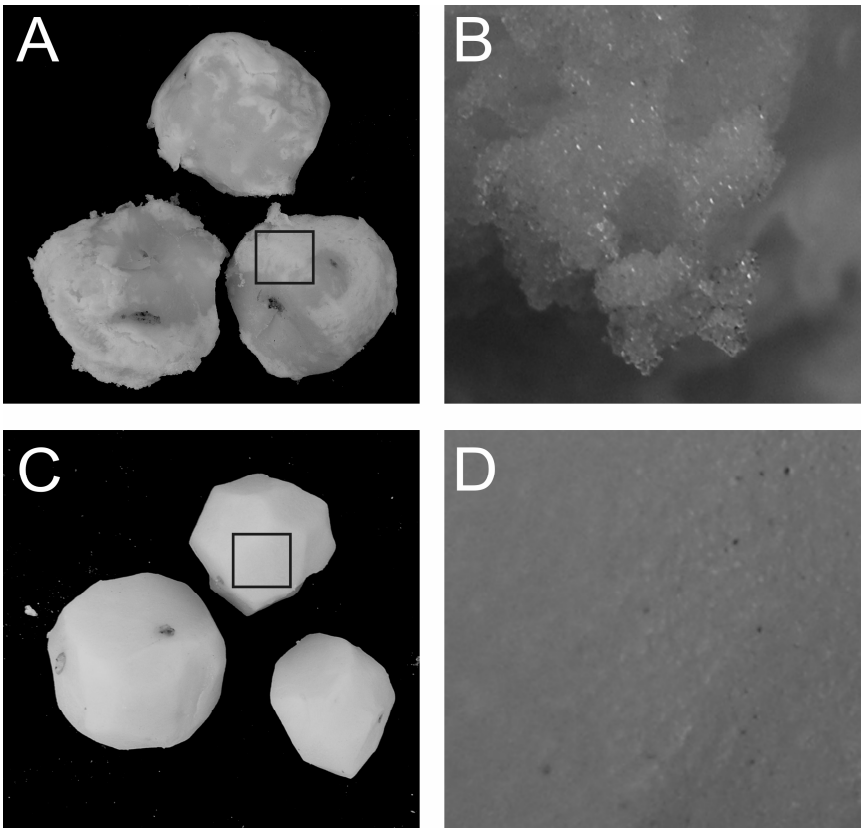

Figure 1; (A-D) Photographs of steam cooked potato tubers of mealy and non-mealy/firm tubers. Three replicates of steam cooked potato tubers of two representative genotypes (CxE), for either a mealy (A) or a non-mealy (C) cooking type. Enlargements of part of the tuber surface of tubers showing a mealy (C) and a non-mealy (D) phenotype.

Bulked expression analysis for tuber texture after cooking

To identify candidate genes involved in the determination of texture after cooking we analyzed gene expression within a subset of genotypes from the CxE population. In a large scale greenhouse experiment 90 individuals of the CxE population, segregating for a number of different tuber quality traits, were grown in five repeats and regularly scored for tuber formation and harvested at different intervals. One of the most predominant phenotypes within the CxE population is the difference in earliness resulting in different time points of

tuber formation throughout the growing season (Celis-Gamboa *et al.*, 2003). To circumvent the problem of analyzing gene expression in tubers that represent differences in tuber physiological age and therefore metabolic status, only significantly sized (>2cm) tubers from plants that have had at least a four week period of tuber growth were used. In addition, a pooling strategy was implemented to further reduce the variability between individual genotypes based on their different plant stature or other tuber qualities allowing the detection of differentially expressed genes that are associated with after cooking texture characteristics. For expression analysis, mRNA from harvested tubers of ten individuals at both ends of the texture after cooking type spectrum were pooled in two bulks representing either a mealy or a non-mealy tuber cooking type (Figure 2). The two bulks, for either a mealy (m) or non-mealy (nm) cooking type were named bulk A(m) and A(nm) respectively, and the number of individuals from each of the different texture classes selected for either bulk are indicated in figure 2.

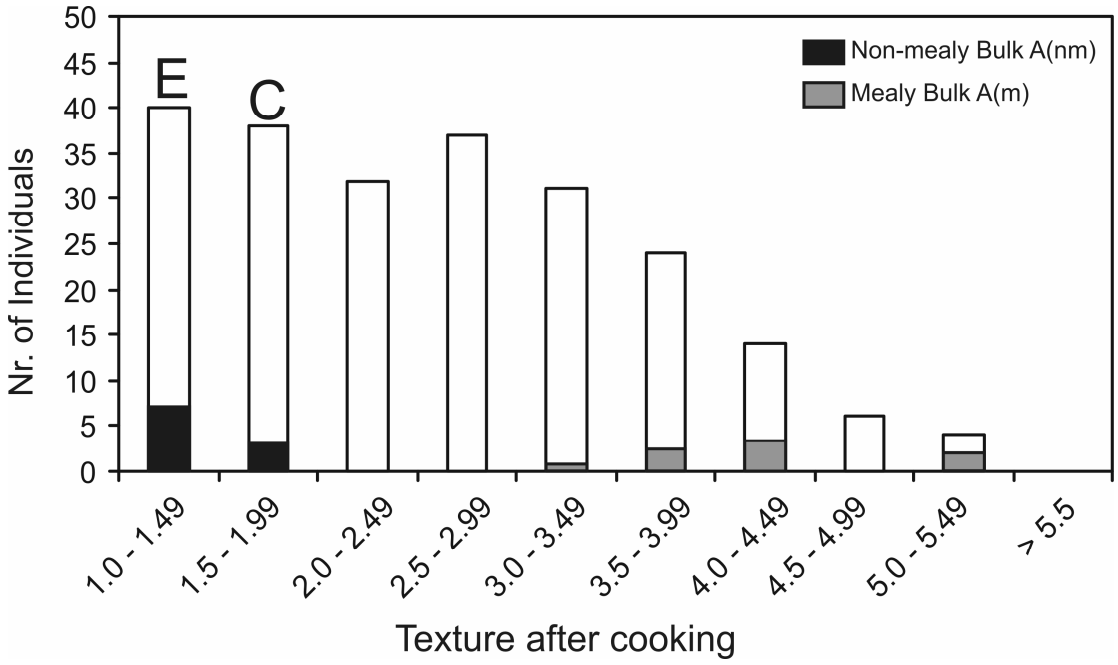

Figure 2; Distribution of the number of individuals from a diploid backcross population (CxE) over the different classes of texture after cooking with values ranging from non-mealy/firm (1) to mealy/crumbly (6). Values are the average of three independent replicates. The total number of genotypes selected for bulk segregant analysis (bulk A) for either the mealy or non-mealy bulk are indicated for each of the classes.

To allow the detection of more subtle changes in expression levels we reduced the initial bulk size and divided bulk A(m) and A(nm), each containing ten individuals, into two separate pools of five non-overlapping individuals into bulk B(m) and B(nm) and bulk C(m) and C(nm), respectively. Using a dedicated potato cDNA microarray, specifically designed for studying tuber development and tuber quality traits and containing a large number of cell wall synthesis and maintenance genes, relative expression levels were obtained by hybridizing the labelled target samples representing the mealy bulks (A(m), B(m) and C(m)) against the corresponding non-mealy bulks (A(nm), B(nm) and C(nm)). For all hybridizations

a swap-dye experiment was included and obtained expression data was normalized and analyzed as described in Experimental procedures. Genes that were consistently and significantly higher expressed (>2-fold) in either the mealy or non-mealy tuber bulks are listed in Table I.

Only three genes showed consistent differential expression patterns across the three bulks indicating a putative correlation between texture after cooking and the level of gene expression. From these, only a single candidate gene showed an on average higher expression in the non-mealy bulks in comparison to the mealy bulks. The differentially expressed gene, represented by cDNA clone (BG096637) exhibited strong sequence similarity to a tomato extra cellular cell wall protein (TLRP). Interestingly, the observed differential expression of the gene is much larger (25-fold) in bulks C in comparison to the expression ratio's found in bulks A and B (2.2 and 2.5-fold, respectively). This observation may indicate that the genotypes represented in both the mealy and non-mealy C bulks (C(m) and C(nm)) have either a more similar and consistent high or low expression level, in comparison to the other bulks, increasing the expression difference.

Table I: Genes with significant differential expression between contrasting bulks for potato tuber cooking type

cDNA clone GenBank accessions	Functional Homology	Median[1] Fold-change across bulks		
		A[2]	B[3]	C[4]
BG096637	Tomato extra cellular matrix protein (TLRP)	2.2	2.5	25.1
BE919835	chlorophyll a/b binding protein type I	-3.4	-4.9	-3.2
BE920360	chlorophyll a/b binding protein type I	-2.6	-3.1	-2.6

[1] Median values were calculated from 2 independent hybridizations including a swap dye and replicates on the array.
[2] Fold difference in relative gene expression levels between bulks A(m) and A(nm) each containing ten individuals from the CxE population exhibiting either a mealy (m) or non-mealy (nm) cooking type
[3] Fold difference in relative gene expression levels between bulks B(m) and B(nm) each containing 5 individuals that were also part of the A bulks
[4] Fold difference in relative expression levels between bulks C(m) and C(nm) each containing 5 different genotypes that were part of the mealy and non-mealy A bulks but non-overlapping with the individuals selected for the B bulks

The two other differentially expressed genes (BE919835, BE920360), with sequence homology to chlorophyll a/b binding proteins (CAB), show on average a higher expression in the mealy bulks. However, based on their putative function and the finding that both these genes were also found to be differentially expressed in bulks for other unrelated tuber quality traits (data not shown), they are considered to be false positives as a result of very strong expression in only a small number of individuals that skew the average expression levels within the bulk. A likely explanation for the identification of this false positive, might be the

growth conditions, since the potato plants were grown in pots, tuber formation occurs relatively close to the surface and a small subset of the harvested tubers may have been directly exposed to light, inducing strong expression of the genes that are part of the light harvesting complex.

It is well documented that the cell wall has an important role in determining potato tuber texture characteristics, and therefore, the identification of a potato EST with high sequence similarity to an extra cellular cell wall protein, that is more highly expressed in the tuber bulk with a more firm/non-mealy tuber texture, is considered to be a promising candidate gene and is analyzed in greater detail.

Sequence analysis and identification of an allelic variant of StTLRP

The full length sequence of the identified candidate gene was obtained by sequencing EST clone (BG096637) derived from a leaf cDNA library of potato *var*. Kennebec. Sequence analysis revealed an open reading frame (ORF) of 237 nucleotides with a predicted protein of 78 amino acids, which showed high sequence similarity (69.3%) with the tomato (*S. lycopersicum*) tyrosine and lysine rich protein (TLRP; X77373). The predicted potato protein is characterized by a high level of tyrosine (7) and lysine (7) residues as well as the presence of a highly conserved N-terminus signal peptide targeting the protein to the extra cellular cell wall matrix (Figure 3A). Based on these observations, the identified gene was designated *StTLRP* and is to our knowledge the first identified TLRP homolog in potato. The predicted potato TLRP protein is smaller than the tomato TLRP due to the absence of two stretches of 3 and 7 amino acids, the latter disrupting a potential Cys domain (CD) that was identified in a tobacco TLRP protein (NtTLRP; CAB67122), and thought to be involved in cross-linking soluble proteins to the cell wall making them insoluble (Domingo *et al.*, 1999).

To allow the design of *StTLRP* specific primers for qRT-PCR analysis of individuals of the CxE population, we first needed to obtain the StTLRP sequences of both parental clones to account for any allelelic variations. Using *StTLRP* specific primers (F1/R1), single bands were amplified for both parents using cDNA templates obtained from C and E growing tubers (Figure 3C). The amplified product in the C parent was slightly larger (372bp) in comparison to the E-parent (348bp). Sequence analysis of the amplified PCR product in the E-parent revealed a predicted ORF and protein sequence that was identical to the identified StTLRP (78 amino acids). Interestingly, within the slightly larger amplified PCR product of the C-parent two different sequences could be identified, having few nucleotide substitutions in the 5'UTR, however, giving rise to identical predicted protein sequences of 85 amino acids in length. Sequence alignment of the predicted protein sequences of both parental lines show that the larger sequence found in the C-parent is the result of the presence of an additional 7 amino acids stretch identical to the sequence present in the tomato TLRP (Figure 3A).

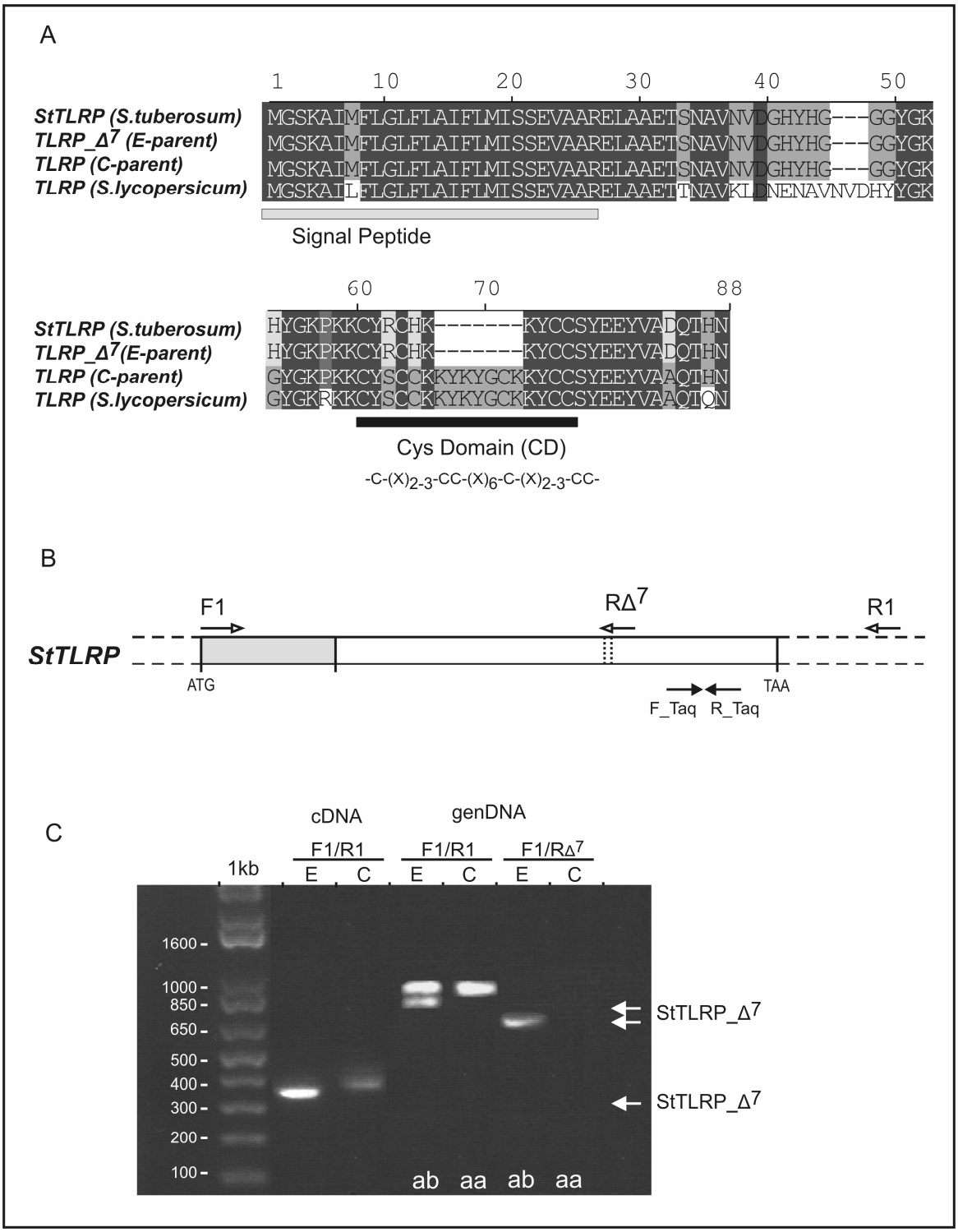

Figure 3; (A) Sequence alignments of predicted StTLRP protein sequence in potato (*S. tuberosum*) including, the identified StTLRP protein, the tomato TLRP protein (CAA54561), and TLRP protein predictions of both potato clones C and E. The TLRP protein in the E-parent was identified as an allelic variant and is therefore identified as TLRP_Δ7. (B) Graphical overview of StTLRP sequence with predicted start and stop codon and signal peptide indicated with a grey box. Relative positions of primers for amplifying either the entire *StTLRP* gene (F1/R1) or TLRP_Δ7 (F1/R_ Δ7) are indicated. StTLRP specific primers for quantitative RT-PCR analysis are also indicated (F_Taq/R_Taq). (C) PCR amplification products for StTLRP on C and E genomic or cDNA using the primer combinations as indicated in (B). StTLRP_ Δ7 allele specific amplification bands are indicated with white arrows. For the genomic DNA samples, absence (aa) or presence (ab) of allele TLRP_ Δ7 are indicated.

To further investigate this difference in amplification products in both parental lines, specific primers (F1/RΔ^7) were designed to only amplify the TLRP sequence containing the 7 amino acid deletion site (TLRP_Δ^7). An amplified product using these gene specific primers was only detected in the E parent in both genomic (Figure 3C) and cDNA templates (data not shown), suggesting the identified StTLRP gene is actually an allelic variant with a 7 amino acid deletion and is hereafter referred to as TLRP_Δ^7. Using the *StTLRP* primers (F1/R1) on genomic DNA, a sequence region spanning the entire ORF and parts of the 3'UTR was amplified from genomic DNA of both parental clones (Figure 3B,C) . Here, two amplified bands (989bp and 852bp) were detected in the E parent while only a single amplification band (989bp) was found in the C parent. Sequence analysis revealed that the lower band (852bp) within the E parent corresponded to the identified allele, TLRP_Δ^7, which showed an additional deletion site of around 115bp present within the identified intron of the StTLRP genomic sequence.

Based on a conserved region of the *StTLRP* gene in the C and E lines, primers were designed (Figure 3B; F_Taq/R_Taq) to measure expression of *StTLRP* in tubers of CxE genotypes with quantitative RT-PCR. Gene expression levels of 56 genotypes, including the individuals represented in the bulks, were measured and showed remarkable variation relative to the internal control (Figure 4). More specifically, all genotypes harbouring the TLRP_Δ^7 allele, showed on average a much higher level of expression in comparison to genotypes lacking the respective allele. Both parental lines show a large difference in expression levels, E has a ΔCt of 0.11 and C a ΔCt of 6.11 which translates to a ($2^{-\Delta\Delta Ct}$) 64-fold higher expression of *StTLRP* in the E-clone. Despite the observed differences in expression levels of *StTLRP* in both parental lines, with a higher expression found in E, both exhibit a relatively non-mealy texture profile indicating that other factors or genes are likely to be involved in regulating potato tuber cooking type within the CxE population. To further investigate a possible correlation between presence or absence of the TLRP_Δ^7 allele and potato tuber cooking type, the distribution of the individual genotypes over the different texture classes was re-examined based on TLRP_Δ^7 scores (Figure 5). In general, genotypes harbouring the TLRP_Δ^7 allele appear to have a more firm/non-mealy texture profile while the distribution of the genotypes lacking the TLRP_Δ^7 allele, shows a shift towards a more mealy tuber texture. However, a substantial number of genotypes lacking the TLRP_Δ^7 allele do exhibit a firm/non-mealy texture indicating that the presence of TLRP_Δ7 is not strictly required for producing a firm/non-mealy phenotype. In fact, the opposite may be true, in which absence of the TLRP_Δ^7 allele is required to produce a mealy phenotype, since it was observed that 34 of the 40 mealiest genotypes lack the identified TLRP_Δ7 allele.

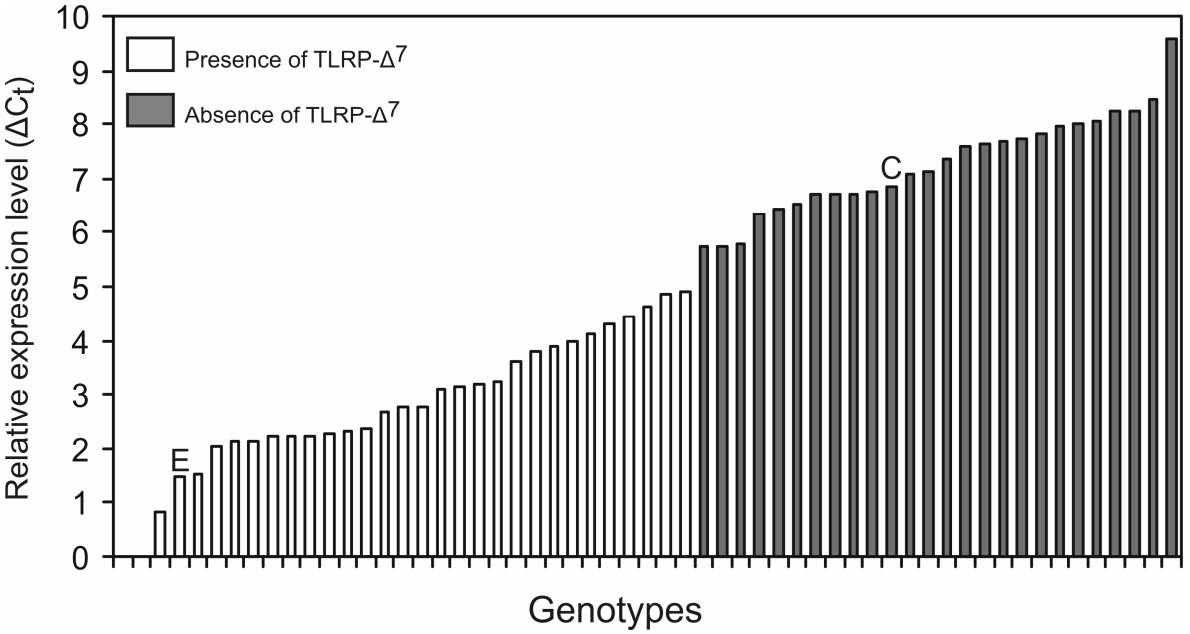

Figure 4; Expression levels of *StTLRP* in 56 genotypes of the CxE population including both parental lines, relative to the internal control (ΔCt). Genotypes were plotted starting from the highest expression level detected (ΔCt=0) and positioned accordingly. Presence of the TLRP_Δ7 allele was scored for each genotype and indicated as follows: presence (ab) white bar and absence (aa) grey bar. Relative expression levels were averaged from three replicated measurements.

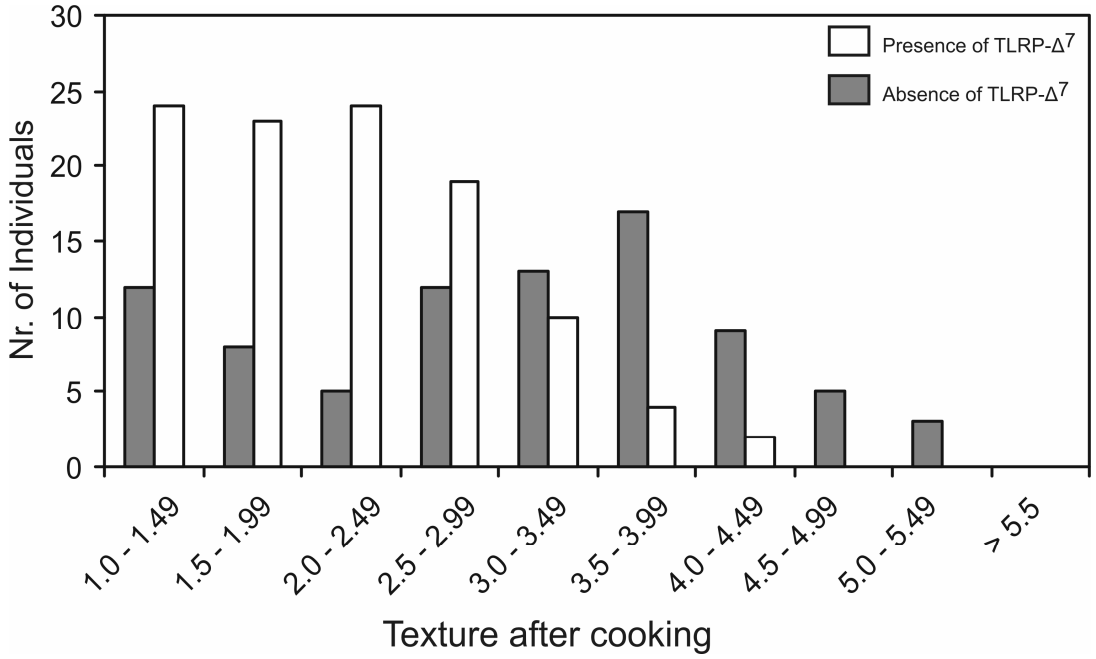

Figure 5; Distribution of the number of individuals from the diploid backcross population (CxE) over the different classes of tuber texture after cooking, ranging from firm/non-mealy (1) to mealy/crumbly (6) divided in two groups based on the presence (white bars) or absence (grey bars) of allele TLRP_ Δ7.

QTL analysis of potato tuber cooking type and StTLRP expression

The identified allelic variant of *StTLRP* present in the E-parent and absent in the C-parent, shows a clear segregation in the offspring in a cross between C and E. Presence (ab) or absence (aa) of TLRP_Δ7 was scored within 209 individuals resulting in a 114 (ab) : 95 (aa) ratio resembling the expected 1:1 Mendelian segregation. Allelic scores were used to integrate the StTLRP marker into an existing genetic map of CxE (Celis-Gamboa, 2002) and was subsequently mapped on the long arm of chromosome 9 of the E-parent (48cM) and positioned between markers E32M49-168e9 (37.8cM) and E32M48-274.9e9 (53.0cM) (Figure 6A).

To examine a possible correlation between *StTLRP* expression levels and tuber texture after cooking, QTL analysis on the texture data and expression levels of candidate gene *StTLRP* was performed. Firstly, interval mapping using the average texture data over the two harvest years of 226 genotypes yielded a single significant QTL (LOD 7.51) on the long arm of chromosome E9 as shown in Figure 6B.

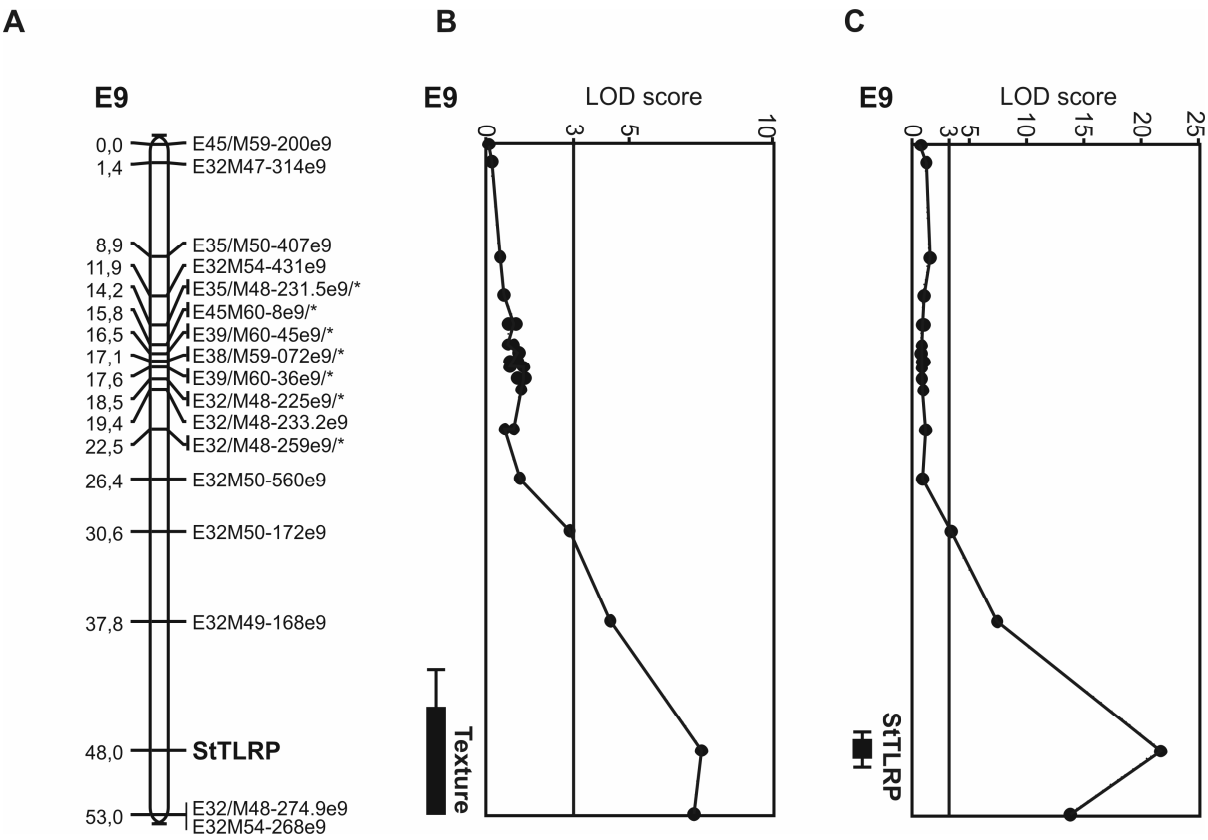

Figure 6; Genetic map of chromosome 9 of the E parent (E9) and QTLs for potato tuber texture after cooking and StTLRP expression. (A) Graphical representation of the genetic map of chromosome E9 with distances of the individual markers indicated (cM). For readability additional markers with identical map positions are indicated with an asterisk (*). LOD scores for potato tuber texture after cooking (B) and StTLRP expression levels (C) are plotted relative to the marker positions on chromosome E9 (dashed line). Minimum significant threshold level (LOD 3.0) is indicated with a black line. Predicted inner and outer QTL interval regions are indicated with black bars on the left hand side of the QTL plots.

The predicted QTL on chromosome 9 of the E-clone explained 14.3% of the observed variance within the combined harvest years. Genome wide significant thresholds for maximum LOD scores were calculated after performing a permutation test and was set at a LOD of 3.0 (p=0.05). The texture QTL shows the highest association with the StTLRP marker in the non-parametric Kruskal-Wallis test (K*= 29.451 with 1df and p-value < 0.0001).

Secondly, the relative expression levels of the identified *StTLRP* gene in potato tubers of 56 individuals was treated as a quantitative trait and interval mapping produced a single strong expression QTL (eQTL; LOD 21.6) positioned directly above the StTLRP marker on the genetic map. (Figure 6C). The high association of *StTLRP* expression levels with the *StTLRP* genetic marker (K*= 39.55 with 1df and p-value 0.0001) on the same chromosomal location, strongly indicates cis-acting transcriptional regulation of *StTLRP*. Moreover, the identified eQTL for *StTLRP* co-localizes with the QTL for texture after cooking providing strong evidence for the association of *StTLRP* expression levels or allelic variation with the texture of cooked potatoes.

Discussion

In this study we have implemented a genetical genomics approach in an attempt to identify novel candidate genes for textural changes in steam cooked potato tubers by using microarray technology. Since hybridization of each individual of the CxE population on a microarray would be very costly and time consuming we have pooled individuals to reduce the number of hybridizations as suggested by Jansen and Nap (2001) and Kerr (2003). The efficiency and reliability of using a pooling strategy for studying gene expression has been tested in a number of studies (Agrawal *et al.*, 2002; Kendziorski *et al.*, 2003; Glass *et al.*, 2005) and has shown to be a valid method for reducing the number of microarray hybridizations, however their experiments did not encompass segregating plant populations. Pooling of genotypes that share a phenotype but are otherwise genetically variable masks expression differences that may be a result of environmental conditions or other variables. The selection of individuals for bulks exhibiting extreme phenotypes unrelated to the trait of interest may, however, result in the identification of false positives. Hence, choice of bulk size is vital when attempting to minimize the number of false positives without loosing too much sensitivity in order to allow detection of more subtle changes in gene expression. Furthermore, the best time point for harvesting and analyzing gene expression in potato tubers is difficult to determine if one does not know at which developmental stage a quality trait, like cooking type, is being established. In a previous study we have shown that gene expression is highly variable during the initial stages of potato tuber development and often stabilizes during the stages of tuber filling before entering a state of dormancy (Kloosterman *et al.*, 2005). In a pilot study of 8 individuals of the CxE population, gene expression was measured for each individual on two separate time points representing actively growing tubers and fully matured dormant tubers. Besides a much larger percentage of genes on the array being more highly expressed in the active growing tubers in comparison to the dormant

tubers, the level of expression variance was also much larger in the former (Vorst *et al.*, unpublished results). Therefore, we have chosen to study gene expression in tubers of a similar developmental stage that are however still active in their tuber filling stage, allowing the detection of a large number of expressed genes. For other quality traits however, one could decide to study either dormant tubers or younger tubers if expected expression differences at these stages would be vital in determining a particular quality trait.

Based on the results from our microarray experiments we have identified a promising candidate gene (*StTLRP*) for involvement in the observed textural changes in tubers after cooking. In total three genes were identified that were consistently differentially expressed in all three bulks analyzed. We found a strong correlation between expression levels of the most promising candidate gene (*StTLRP*) with potato tuber cooking type as exemplified by the co-localization of the eQTL and the QTL for texture on chromosome E9 (Figure 6A-C). In addition, sequence analysis allowed the identification of an allelic member of *StTLRP* that was shown to be responsible for the high expression levels and was mapped to the same genetic map location (Figure 6A). The observation that the *StTLRP* genetic map position co-localizes with the identified expression QTL indicates cis-acting transcriptional control. The precise mechanism controlling the level of *StTLRP* expression is however still unknown.
StTLRP was named after its high homology with a class of extra cellular cell wall matrix proteins identified in tomato (TLRP; Domingo *et al.*, 1994) and tobacco (NtTLRP; Domingo *et al.*, 1999). This class of cell wall proteins is characterized by high level of tyrosine and lysine residues and a highly conserved N-terminus signal peptide targeting the protein to the cell wall. These proteins are thought to be involved in cross-linking other proteins to the cell wall making them insoluble (Domingo *et al.*, 1999). Insolubilization of plant cell wall proteins rich in tyrosine residues has been well documented and seems to be based on the oxidative coupling of two tyrosine molecules connected through a diphenyl ether bridge (Fry, 1982). Insolubilization capacity of the TLRP proteins is thought to be associated with the presence of a cysteine-rich domain (CD-domain) in which the conserved arrangement of cysteine residues allows the formation of intermolecular cysteine bridges, that may stabilize an appropriate conformation so that the other residues (including tyrosine) will be exposed and thus facilitates interaction with other components of the cell wall (Domingo *et al.*, 1999). In tobacco, it was shown that the CD-domain is sufficient to cross-link previously soluble proteins to the secondary cell wall (Domingo *et al.*, 1999). Since the mass of potato tubers consists largely of storage parenchymatous cells that do not contain a secondary cell wall, it will be important to determine the cell specificity and localization of the StTLRP protein within the cell wall. In this respect, an interesting observation was made by Domingo *et al*. (1994), that although the TLRP in tomato showed highly localized deposition in the secondary cell walls of lignified cells, low levels were also detected in intact primary walls of parenchymatous cells. Also in other related classes of cell wall proteins including, the hydroxyproline-rich glycoproteins (HRGPs), the proline-rich proteins (PRP) and the glycine-rich proteins (GRPs), gene expression is tightly regulated and show different cell type specificities and developmental regulation (Keller, 1993; Showalter, 1993).

Studies on potato cell walls before and after cooking have shown that the degree and speed of cell wall solubilization, particularly the middle lamella is important in determining the level of cell adhesion generally regarded as the primary component responsible for the observed cooking types (van Marle, 1997). It is therefore, interesting to postulate a role for StTLRP in modifying cell wall characteristics by cross-linking specific proteins to the extra cellular matrix making the entire cell wall complex more resistant or susceptible to breakdown during cooking. Interestingly, the identified StTLRP lacks a stretch of 7 amino acids in comparison to the tomato TLRP disrupting a likely CD domain thereby potentially altering StTLRP protein conformation and capacity to bind previously insoluble proteins to the cell wall (Figure 3A). The presence of this deletion site within a potential CD domain, appears to be an allelic variant within the CxE population giving rise to genetic and potentially phenotypic variability.

Expression levels of other cell wall enzymes and proteins that have been associated with modifying cell wall characteristics that are represented on our dedicated microarray, including pectin methyl esterases, xylosidases, endoglucanases, expansins and other structural proteins, did not show any correlation with potato tuber cooking type within in the tested bulks. Of course we realize that not all genes or gene family members associated with cell wall assembly or wall modifications were represented on our dedicated array and therefore these and other genes may still fulfil pivotal roles in determining potato tuber cooking type within C x E.

Many questions remain open on how the identified StTLRP can influence the observed textural changes in tubers after cooking within the CxE population. Not only were we able to identify differences in transcript levels that may lead to elevated protein levels and therefore altered tuber quality traits, the identification of an allelic variant coding for a smaller protein with potentially altered protein stability or function may also result in altered tuber characteristics. Interestingly, there appears to be a stronger association between the absence of the high expresser allele TLRP_Δ7 in extreme mealy tubers than presence of the same allele in firm/non-mealy tubers (Figure 5). This observation indicates that the other non-identified *StTLRP* allele(s) coming from the C and E parent may be to a large extent responsible for the observed mealy tuber textures. Genomic sequence analysis of amplified PCR products using StTLRP specific primers as well as Southern blotting with *StTLRP* cDNA as probe, have shown that the identified *StTLRP* is most likely part of a gene family with highly homologous members, making it difficult to identify the other allelic constituents (Kloosterman, data not shown). Nevertheless, the identification of other alleles present within the CxE population should provide a better picture of the genetic components underlying *StTLRP* expression and function in relation to potato tuber cooking type. Furthermore, implementing reverse genetic approaches could provide supporting evidence for a role of StTLRP in determining potato tuber cooking type.

By using a mRNA pooling strategy we were able to identify a novel candidate gene for potato tuber cooking type (*StTLRP*). This approach is an acceptable and more cost-effective alternative to hybridizing individuals and can be easily copied for other potato tuber

quality traits for which no likely candidate genes have been identified and a segregating population with reliable phenotypic data for the trait of interest is available.

Experimental procedures

Plant Material

The diploid backcross population (CxE) consisting in total of 251 individuals was obtained from the cross between C (USW5337.3) and E (77.2102.37). Clone C is a hybrid between *S. phureja* and *S. tuberosum* dihaploid USW42. Clone E is the result of a cross between clone C and the *S. vernei-S. tuberosum* backcross clone $VH^3$4211. Textural changes of tubers after cooking were determined on two consecutive harvests (1998, 1999). Harvested tubers derived from field experiments with three replicates for each genotype, each consisting of two plants. Tubers of the three replicates were harvested and stored for three weeks in controlled conditions before being analyzed. Three tubers of each sample were peeled and steam cooked for 20 minutes after which texture was visually scored on a nominal scale ranging from 1 (firm/non-mealy) to 6 (extreme mealy). A total of 226 genotypes were scored for each of the three harvests and averaged for each year separately. An averaged texture value for each genotype was calculated from the two harvest years and used in QTL analysis. All QTL analysis was performed using the software package MapQTL® 5.0 (Van Ooijen, 2004). For expression studies, sprouting tubers of 94 CxE individuals, including both parent lines, were potted in five replicates in 5L soil-filled pots in the greenhouse and grown for 3 months. Plants were regularly scored for tuber formation and tubers (>2cm) were harvested from a single plants at around 2½ week intervals and immediately frozen in liquid N_2. A single tuber harvest of a subset of the genotypes, that represent a similar developmental stage having had a period of four weeks of tuber growth, were selected for gene expression studies (56 genotypes).

RNA-isolation and microarray hybridization

Total RNA was isolated from tubers as described by Bachem *et al.,* (1996). mRNA was purified using the GenElute™ mRNA miniprep kit (Sigma Aldrich, Zwijnberg, the Netherlands) and mRNA quality and quantity was checked using the NanoDrop ND100 (NanoDrop Technologies, Wilmington, Delaware USA). Equal amounts of purified mRNA from the selected individuals were pooled in bulks of ten genotypes for either mealy or non-mealy tuber characteristics, bulk A(m) and bulk A(nm) respectively. Similarly, four bulks of five non-overlapping genotypes were made, bulk B(m), B(nm), C(m) and C(nm). All bulks were prepared in two repeats and first strand cDNA synthesis followed by target labelling with either the Cy3- or Cy5-dye (Amersham BioSciences, Roosendaal, the Netherlands) was performed using the SuperScript™ Indirect cDNA Labelling System (Invitrogen, the Netherlands). Expression levels of genes within the selected bulks differing in textural changes after cooking were determined relative to one another in a swap dye experiment

using the dedicated potato cDNA-array (Kloosterman *et al.*, 2005). Microarray slides were pre-hybridized and processed as described in van Doorn *et al*., (2003). Hybridization of the target samples was performed in the HybArray12™ (Perkin Elmer, Niewerkerk, the Netherlands) hybridization station at 42˚C over a period of 20 hours. Following hybridization, slides were washed as described in van Doorn *et al*. (2003). Slides were immediately scanned using a Scanarray®ExpressHT scanner according to the manufactures specifications (Perkin Elmer, Niewerkerk, the Netherlands). Spotfinding, data extraction, LOWESS normalization and several quality control filters (Spot quality, Low intensity threshold, Signal to Noise Ratio) were performed with the ScanArray® (Perkin Elmer, Niewerkerk, the Netherlands) and the Microsoft® Excel software package. Detection of significantly differentially expressed genes shared between both suppression clones was performed using the SAM software (Tusher *et al.*, 2001) with a set FDR of 0 and minimal 2-fold expression difference in all three bulks analyzed. The identified differentially expressed gene set was imported into the SPSS software package and were subjected to T-testing ($p<0.005$) for significant expression change >2-fold in both directions.

DNA Sequence analysis

The full length sequence of the potato *StTLRP* was obtained by sequencing cDNA clone BG096637 deriving from a potato leaf EST library (cv. Kennebec) using vector based primers T3 and T7. All sequence data was analyzed with the Vector NTI suite (Informax, Bethesda, USA). Homology searches were carried out using the blastX program (Altschul *et al.*, 1997). Protein alignments were carried out using the ClustalX program (Thompson *et al.*, 1997).

Quantitative RT-PCR

Relative expression levels of the TLRP gene were determined by real-time quantitative reverse transcriptase PCR (qRT-PCR) on a Perkin Elmer Abi Prism 7700 Sequence detector (Perkin Elmer, Niewerkerk, the Netherlands) following the protocol described in 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: F_Taq forward primer 5'-TCCAATGCGGTAAACGTTGA-3', R-Taq reverse primer 5'-CTTACCATAGCCGCCACCAT-3' and *ubi3* (L22576) forward primer 5'- TTCCGACACCATCGACAATGT-3', reverse primer 5'-CGACCATCCTCAAGCTGCTT-3'.

Genetic map and QTL analysis

To score presence of allele TLRP_Δ7 within the CxE population including both parents (C and E), PCR was performed on genomic DNA and cDNA using the following

primer sequences: F1: 5'-ATGGGTTCCAAGGCAATTATGTT-3', R1: 5'-GAATGGCTTTATTCATACTTGTT-3', RΔ[7]: 5'-GCAGCAGTATTTTTGTGGCAT-3'. Using primers F1 and R1, 209 genotypes were scored for presence (ab) or absence (aa) of allele TLRP_Δ7. Integration of StTLRP marker in the existing genetic map of CxE (Celis-Gamboa, 2002) was performed using the software program JoinMap 3.0 (Van Ooijen and Voorrips, 2001).

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Chapter 6

General Discussion

General Discussion

Introduction

The aim of the research described in this thesis was to implement a relatively new genomic tool (i.e. microarray technology) in the ongoing studies to understand the process of potato tuber development and the mechanisms underlying differences in tuber quality traits. A better understanding of how these processes are regulated on a genetic level, through identification of the key regulatory genes involved, will greatly enhance the capabilities for crop improvement through marker assisted breeding methods or genetic engineering. The results obtained and described in this thesis will be discussed with respect to the above mentioned aim and the continuing advancement and importance of implementing large scale genomic tools in the field of potato research.

Construction of a dedicated cDNA-microarray

Microarray technology was developed in the mid 90's with the arraying or spotting of a number of complementary DNA's (cDNA) representing different genes that allowed the simultaneous detection of individual expression levels using two-colour fluorescence hybridizations (Schena *et al.*, 1995; Schena, 1996). The enormous potential of this method was immediately recognized and the use of microarray based profiling quickly spread throughout the scientific community covering the different areas of genome research providing new leads in almost all fields of biology (Schena *et al.*, 1995; DeRisi *et al.*, 1996; Lashkari *et al.*, 1997). The identification of an expressed gene in strawberry involved in determining flavour of ripening fruit showed the potential of cDNA microarrays in discovering novel regulatory genes in yet undefined metabolic pathways in plants (Aharoni *et al.*, 2000). Through the continuing development of arraying capabilities and the completion of the genome sequence of *Arabidopsis thaliana*, whole genome arrays are now available for this model plant. In order to allow researchers to compare gene expression data across experiments, publicly accessible expression databases were constructed enhancing data and knowledge exchange (Rhee *et al.*, 2003; Zimmermann *et al.*, 2004). With perhaps the exception of rice, a similar rapid technological advancement for many other crop species is not yet possible. However, with the rapidly decreasing genome sequencing costs, this will only be a matter of time.

In potato research, large scale transcriptomics analysis for the identification of regulatory genes involved in the process of potato tuber development relied primarily on cDNA-AFLP technology (Bachem *et al.*, 1996). This technology proved to be a valuable tool in the identification of candidate genes based on sequence polymorphisms and expression changes of expressed genes during the process of *in vitro* tuberization (Bachem *et al.*, 1996; Bachem *et al.*, 2000a). However, despite the detection of over 250 differentially expressed fragments that have resulted in a number of interesting candidate genes (Bachem *et al.*, 2000b; Bachem *et al.*, 2001), large scale expression analysis of genes linked to metabolic or

signalling pathways, thus providing a more complete overview has been lacking. Furthermore, cDNA-AFLP studies are extremely laborious if one attempts to study differential expression within the same developmental process under various climate or growth conditions. In contrast, cDNA-microarray technology offers the possibility to study the expression levels of a large set of genes at consecutive time points, thereby allowing multiple repeats and easy comparison across experiments potentially providing novel insights into gene regulation and helping to identify genes involved in the regulation of changes in metabolic pathway fluxes. Although no sequence information is required for designing cDNA-microarrays, since cDNAs can be randomly cloned from any tissue or developmental stage and spotted on glass slides, the availability of large EST databases provides additional information on putative gene function based on sequence similarity with genes available in public databases.

To study potato tuber development and tuber quality traits in a more efficient and high through-put manner, it was decided to construct a dedicated cDNA-microarray (Chapter 2). Based on our research interests, the primary focus was to analyze genes involved in the metabolism of carbohydrates, amino acids, cell wall, hormones, vesicle trafficking, synthesis of secondary metabolites, and genes related to other plant developmental processes. In order to identify as many relevant clones as possible that could be fitted within the dedicated array design, a novel automated text-mining approach was developed. Many of the targeted genes were found and selected from the available unigene set based on the respective blast output. However, not all gene functions selected for representation on the dedicated array are available within the EST databases. Furthermore, since the selection of clones is based on the available literature or established assumptions of gene function covering these topics, such an approach bypasses potentially important genes that are not part in any of the major pathways listed or have not yet been linked to potato tuber development or other related processes in any way. It is therefore important to realize that the potential of novel gene discovery is reduced in such a dedicated array in comparison to a completely random clone selection approach. On the other hand, the nature of random cloning and selection also carries the risk of over-representation of highly expressed genes leading to high gene redundancy on the array.

Although the number of available ESTs has grown substantially since the early database release (August 2001) and hypotheses on mechanisms controlling tuber development and quality traits are continually changing, we consider that the chosen genes remain a relevant and representative set, well suited to study tuber development and associated tuber quality characteristics at the transcriptional level. Furthermore, the constructed text mining approach can be readily implemented for other plant species, processes or functional categories for which a dedicated microarray is desired or where full transcriptome arrays are currently not within the realm of possibilities.

Studying potato tuber development and growth

Potato tuber formation is often regarded as the model system for studying underground sink organ formation. Therefore, understanding of the regulatory elements controlling tuber development and growth with respect to sink-source relationships is extremely important. Despite the identification of a number of regulatory conditions and genes, a coherent sequence of events regulating the formation of an underground tuber has not been established thus far. The visible process of potato tuberization involves the transition of a longitudinal growing stolon into an active sink tuber through cell expansion, followed by longitudinal cell division of the sub-apical stolon region (Xu *et al.*, 1998b). Further tuber growth is primarily due to random cell division and expansion in the perimedullary region (Peterson *et al.*, 1985). Several studies investigating the process of potato tuber formation have successfully used an *in vitro* tuberization system (Koda and Okazawa, 1983; MacIntosh *et al.*, 1996; Banfalvi *et al.*, 1997; Suttle, 1998; Xu *et al.*, 1998a; Bachem *et al.*, 2001; Appeldoorn *et al.*, 2002; Rosin *et al.*, 2003). However, secondary growth of the tuber is completely inhibited in the *in vitro* system, most likely due the absence of an unidentified growth signal within the explants or media (Vreugdenhil and Struik, 1989). Since secondary growth of the tuber is such an important aspect of tuber development and largely determines final tuber size and quality attributes, a synchronous *in vivo* experiment was designed to study both early tuber initiation and secondary growth with the added value of studying whole plant dynamics (Chapter 2). The synchronous *in vivo* tuberization system is an improvement over the *in vitro* tuberization system where tuber induction is primarily induced through the presence of high sucrose levels (Hendriks *et al.*, 1991; Appeldoorn *et al.*, 1997). In the *in viv*o system, synchronous tuber formation was achieved by switching the light conditions from long day (16h) to short day (8h) after which the plants formed tubers within 5-7 days. Day-length, or rather the duration of the dark period, has a major impact on controlling the time point of tuberization. In general, tuberization is promoted by long nights (i.e. short days) with the degree of response largely dependent on genotype and physiological age of the plant (Ewing and Struik, 1992). Most cultivated potato varieties no longer strictly require short days in order to tuberize, however, we found that drastically reducing day length, enhances synchronous tuber formation in similar aged plants. Non-tuberising stolon tips were harvested under both conditions allowing the detection of differentially expressed genes, as a result of the shift in day length. In addition, six developmental stages were harvested including; first visible swelling, tuber initiation, and four tuber growth stages. This set provides an accurate representation of the different developmental stages during potato tuber development reflecting natural conditions as well as whole plant dynamics allowing the detailed study of gene expression changes.

Candidate gene identification and gene function validation

The synchronous *in vivo* system was used to study tuber development and allowed the detection of early, late and temporal changes in gene expression levels. The collective

analysis of all expression profiles of genes represented on the dedicated array resulted in the identification of a number of common expression profiles as presented and discussed in chapter 2 (Kloosterman *et al.*, 2005). The entire dataset of gene expression profiles as well as the data on tissue specificity of the genes represented on the tuber life cycle array is a valuable resource for potato researchers world-wide. Not only can novel candidate genes be identified based on their expression profiles, tissue specificity and putative function, the presence of genes acting within the same metabolic or signalling pathway may advance our understanding of co-regulated gene expression and other transcriptional control mechanisms. For example, many genes directly involved in the sucrose to starch synthesis pathway are strongly up-regulated from the swelling stolon stage onwards (Chapter 2; Figure 3). The genes coding for enzymes directly associated with sucrose cleavage show changes in expression during the early stages of tuber development that correlate with a switch in the predominant sucrose breakdown route and metabolic flux directed towards increased starch synthesis (Appeldoorn *et al.*, 1997; Viola *et al.*, 2001). Similarly, genes that are up-regulated prior to visible swelling after the switch from long day to short day conditions indicate an early role in facilitating tuber initiation whilst genes that are up-regulated after a tuber has been formed are more likely to be involved in tuber filling or supporting additional tuber growth that is important in determining potato tuber quality traits such as: flesh colour, enzymatic browning, cooking type, starch content, and tuber nutritive value. From the same study it was shown that genes, showing strong changes in relative expression levels during tuber development, appear to have a higher degree of tissue specificity. Hence, genes that are uniquely expressed within the stolon tip or tuber are more likely to fulfil a process dependent or specific function, whilst ubiquitously expressed genes tend to be involved in more basic plant functions.

The combined gene information based on the expression profile during tuber development, tissue specificity, and putative function resulted in a number of promising candidate genes involved in tuber development. In order to properly assess gene function and its importance in relation to the developmental process studied, additional experiments are required. In potato, reverse genetic approaches are the most suitable method for assessing gene function since the potato plant is a tetraploid species that is clonally propagated and therefore few mutant lines are available. Due to the laborious and time consuming nature of plant transformation and regeneration experiments, only a small subset of candidate genes were selected for further analysis. Out of this subset of promising candidate genes selected for validation studies, two genes showed strong phenotypic alterations in the respective transgenic clones in comparison to the untransformed control and are presented in chapters 3 and 4. Firstly, a gene (*StGA2ox1*) with homology to a GA2-oxidase was strongly up-regulated after the switch from long day to short day conditions and 35S-over-expression transformant plants exhibit a dwarfed phenotype, reduced stolon length and earlier *in vitro* tuberization. Transgenic plants with reduced expression levels of *StGA2ox1* showed normal plant growth, an altered stolon swelling phenotype and delayed *in vitro* tuberization (Chapter 3). Secondly, a gene (*StIAA2*) with homology to the Aux/IAA family of transcription factors was strongly down-regulated at tuber organogenesis, and down-regulation of *StIAA2*

transcript levels resulted in distinctive phenotypes that include, increased plant height, petiole hyponasty and extreme curvature of growing leaf primordia (Chapter 4). In the latter study, additional transcriptomic analysis of *StIAA2* transgenic clones, using the dedicated cDNA microarray, yielded differential expression of additional genes that may, directly or indirectly, be regulated by StIAA2 (Chapter 4; Table II). The identification of several genes with altered expression levels in transgenic clones exhibiting a particular phenotype shows the strength of microarray technology in revealing transcriptional control mechanisms that may lead to novel biological insights and linking of different signalling pathways.

As is often the case with reverse genetic approaches, not all of the selected candidate genes generated obvious phenotypic changes in transgenic plants where their expression had been altered when compared to the untransformed control plants. Unfortunately, although these genes were selected because of their differential expression during potato tuber development, no additional evidence for a role in potato tuber development or tuber quality was obtained. A more detailed analysis of these transgenic plants may however, reveal evidence of a role for these genes and it can thus not be excluded that they are in involved in potato tuber development and/or tuber quality.

In conclusion, the constructed cDNA-microarray and synchronous tuber developmental system provides a powerful tools for the identification of novel candidate genes and the transcriptional control mechanisms behind them. However, identification of candidate genes based on expression profiles is only a starting point and additional gene function validation studies are needed in order to properly assess candidate gene function.

The role of GA in potato tuber development

Gibberellins (GAs) have long been implicated to play a regulating role in potato stolon growth and tuber initiation (Booth, 1963; Kumar and Wareing, 1974; Ewing, 1987). Applications of biologically active GAs or inhibitors of GA biosynthesis have shown to either delay or promote tuber formation under tuber inducing conditions respectively (Vreugdenhil and Struik, 1989; Jackson and Prat, 1996). Another important piece of evidence, linking GAs to the regulation of tuber development, was the observation of a decrease in the levels of GA_1 within the sub-apical region of *in vitro* grown microtubers prior to visible swelling (Xu *et al.*, 1998a). The regulatory mechanism by which levels of bioactive GAs are reduced prior to stolon swelling is, however unknown. Therefore, the identification of a gene (*StGA2ox1*) with strong homology to a GA catabolic enzyme (GA2-oxidase), exhibiting an early up-regulated expression profile during tuber development, was extremely intriguing and was analyzed further as a candidate gene presented in chapter 3. *StGA2ox1* showed a significant increase of expression levels already after the switch from long day to short day conditions. Transgenic clones over-expressing the *StGA2ox1* gene exhibit a dwarfed phenotype, reduced stolon length and earlier *in vitro* tuberization, whilst transgenic plants with reduced expression levels of *StGA2ox1* showed normal plant growth, an altered stolon swelling phenotype, and delayed *in vitro* tuberization. In addition, the tubers of the silencing clones contain increased levels of GA_{20}, indicating altered GA metabolism. In both

the over-expression and silencing clones, time point of tuber formation in the *in vivo* plants was not significantly altered. However, within the *in vitro* system either a strong induction or delay in the rate of tuber formation was observed for the over-expression and silencing clones respectively (Chapter 3; Figure 8). Under *in vitro* conditions the time point of tuber formation appears to rely primarily on sucrose/GA interactions within the stolon tip (Xu *et al.*, 1998a) whilst under *in vivo* conditions the synthesis and transport of the tuber inducing signal in the leaves or its sensitivity within the stolon tip controls the tuberization time point (Jackson and Prat, 1996). Interestingly, over-expression of a GA synthesis gene, *StGA20ox1,* results in plants that show delayed tuber formation, whilst silencing clones are semi-dwarfed and tuberize earlier under short day conditions, indicating a role for GA in the synthesis or transport of the tuber inducing signal in leaves (Carrera *et al.*, 2000). Since no difference in the time point of tuber formation was observed in the *StGA2ox1* transgenic clones, it is thought that there may be two independent roles for GA in controlling potato tuber development: one in the leaves regulating the synthesis and/or transport of the tuber inducing signal and the other, in the stolon tip facilitating the transition of a stolon into a tuber. Based on these findings we propose a role for StGA2ox1 in regulating GA levels in the stolon at tuber organogenesis, allowing normal tuber development and subsequent growth. It is noteworthy that within our study we also observed a strong decrease in expression levels of a GA3β-hydroxylase gene (*StGA3ox2*), catalyzing the last synthesis step of bioactive GAs, prior to tuber formation. A decrease in GA biosynthesis in the stolon tip would thus lower the levels of active GAs thereby inhibiting longitudinal growth. Interestingly, the up-regulated expression profile of StGA2ox1, catalyzing the inactivation of GAs, could result in a similar decrease of bioactive GAs in the stolon tip. In order to explain the complementary expression profiles of both enzymes, a working hypothesis has been constructed in which StGA2ox1 activity is primarily localized within the sub-apical region of the stolon and growing tuber, facilitating radial swelling and growth, while StGA3ox2 activity is restricted to the stolon apex controlling GA levels regulating longitudinal stolon growth (Figure 1).

At the time point of tuber formation, StGA3ox2 activity diminishes causing a reduction of bioactive GAs in the apex, resulting in the inhibition of longitudinal stolon growth, whilst GA within the subapical region is inactivated through the increased expression and thus 2-oxidation activity of StGA2ox1, resulting in the reorientation of the plane of cell division leading to visible radial swelling. It has been well documented that GA levels promote longitudinal cell division and elongation within plants by affecting the orientation of microtubules and microfibrils (Shibaoka, 1993; Fujino *et al.*, 1995; Sanz *et al.*, 1996). Supporting evidence for such a model however, would necessitate the detection of RNA transcripts using *in situ* hybridization from genes coding for both enzymes. It will be equally important to assess the degree of transport of bioactive GAs or their precursors from the leaves to the stolon tip and their roles in regulating stolon growth and tuber transition. Nevertheless, with the identification and analysis of *StGA2ox1*, novel insights and leads into the role of GA in tuber organogenesis have been obtained.

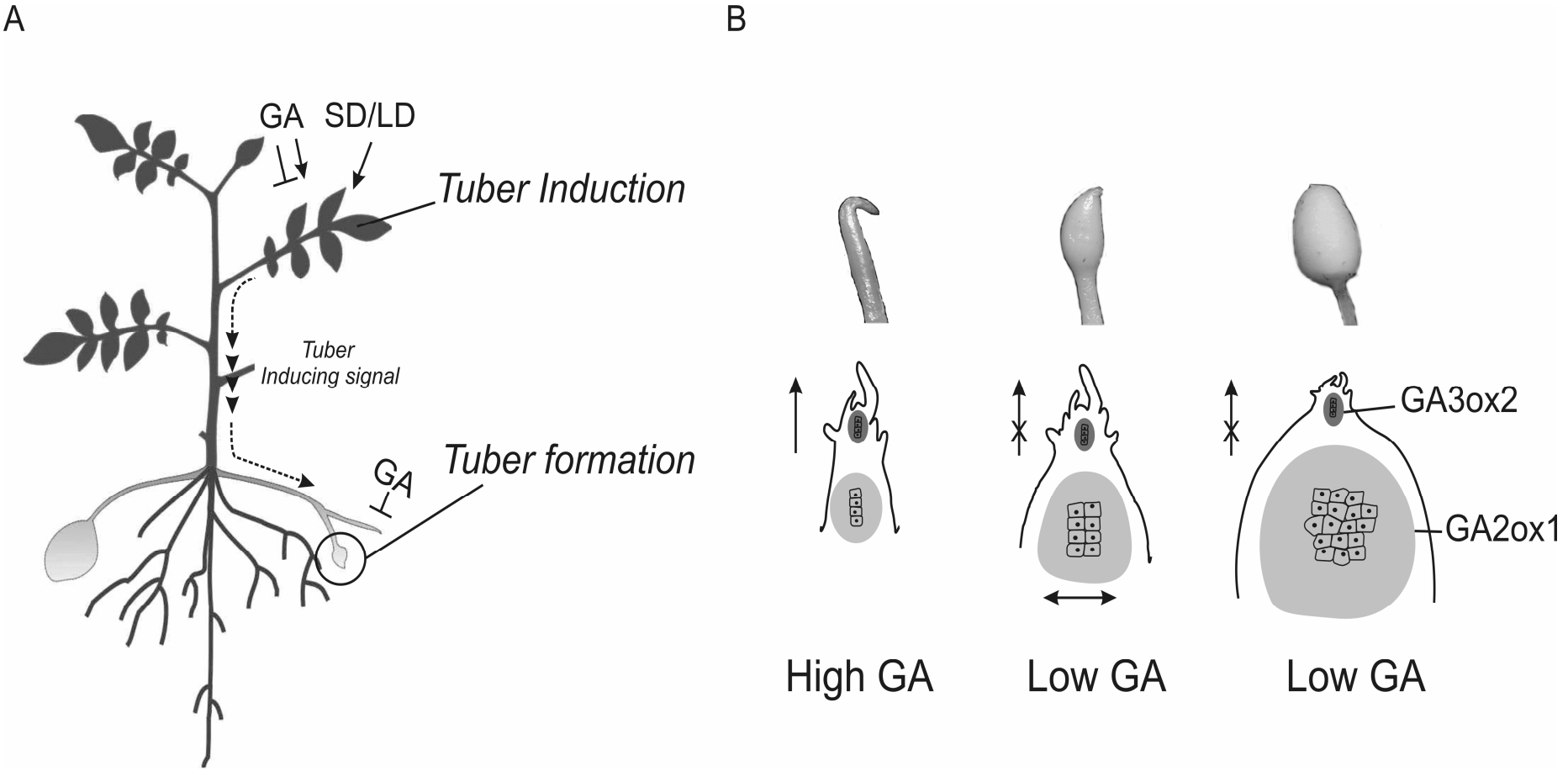

Figure 1; Model of the process of potato tuber formation. (A) Photoperiod is perceived in the leaves and under inducing conditions (i.e. short days) a yet unidentified tuber inducing signal is transported basipetally towards the stolon tip, a process thought to be mediated by GA. (B) Prior to tuber induction, longitudinal stolon growth is promoted by the high levels of GA within the stolon tip. The combined actions of StGA3ox2 and StGA2ox1 expression changes at tuber organogenesis, results in lowered GA levels within the stolon apex (dark grey) and subapical region (light grey) respectively. The reduction of bioactive GAs results in the inhibition of longitudinal stolon growth followed by the reorientation of the plane of cell division, causing radial subapical stolon swelling. Arrows indicate direction of cell division and growth. Secondary tuber growth occurs through randomly orientated cell division in the perimedullary region.

Potato tuber quality and the power of genetical genomics

Unlike a conserved process like potato tuber formation, identification of differentially expressed genes related to individual tuber quality traits is a much more difficult task due to different mechanisms underlying phenotypic variation. Numerous factors are concerned with potato quality and relate to the morphology, structure and chemical composition of the tuber which can be affected by the environment during growth (Gray and Hughes, 1978). Furthermore, the allelic composition of each variety or genotype can have a large impact on final tuber characteristics. Transcriptomic analysis of potato tuber quality traits can expedite the identification of candidate genes through scoring of differential expression between genotypes representing contrasting phenotypes. The use of transcript profiling techniques, such as microarray or cDNA-AFLP technology, for the profiling of individuals within a population has been increasingly used as a valid method for the identification of candidate genes related to a trait of interest (Brem *et al.*, 2002; Wayne and McIntyre, 2002; Schadt *et al.*, 2003; Kirst *et al.*, 2004; Vuylsteke *et al.*, 2006). In addition, by treating mRNA transcript abundances as a quantitative trait, candidate genes may be identified as expression QTLs on genetic maps (Jansen and Nap, 2001; Schadt *et al.*, 2003). The combination of expression profiling and genetics has been referred to as 'genetical genomics' and is expected to greatly advance our capabilities to resolve metabolic, regulatory and developmental pathways (Jansen and Nap, 2001; Li and Burmeister, 2005).

In order to study gene expression in relation to potato tuber quality a diploid segregating population (C x E) was used, segregating for a number of economically important tuber characteristics. In the past, several genetic maps have been constructed for the C x E population (Jacobs *et al.*, 1995; van Eck *et al.*, 1995; Celis-Gamboa, 2002) and the population has been used for the genetic and phenotypic study of the different aspects of the potato tuber life-cycle (van Eck *et al.*, 1994; Celis-Gamboa *et al.*, 2003).

In Chapter 5 we demonstrate the usefulness of the dedicated potato cDNA-microarray in the analysis of gene expression and identification of candidate genes in relation to potato tuber quality traits. Since hybridization of each individual of the C x E population on a microarray would be very costly and time consuming, we have implemented a pooling strategy by combining individuals into groups (referred to as bulks) with contrasting phenotypes relating to the trait under investigation. Genetic or genomic analysis of pools or bulks of individuals within a segregating population that are identical for a particular trait is referred to as bulked segregant analysis (BSA) and diminishes genetically based differences that are of no relevance to the trait of interest (Michelmore *et al.*, 1991). A BSA approach in combination with microarray technology, as presented in Figure 2, allows the identification of differentially expressed genes by comparing bulks with contrasting characteristics and dramatically reduces the number of required hybridizations as predicted by Jansen en Nap (2001) and Kerr (2003).

As an example of the power of such a BSA approach in combination with microarray technology, we have identified a candidate gene (StTLRP) that was differentially expressed within the bulks and individuals of the CxE population exhibiting a variation in the degree of

textural changes after cooking (Chapter 5). The segregation of StTLRP expression levels were treated as a quantitative trait and resulted in the localization of the StTLRP eQTL to the same genetic map position as the QTL representing potato tuber cooking type. *StTLRP* was regarded as a high quality candidate gene based on its expression profile and strong homology to an extra cellular cell wall protein. StTLRP is thought to be involved in cross-linking other proteins to the cell wall thereby altering cell wall composition. Cell wall characteristics have been shown to be responsible for the observed variation in potato tuber cooking types by controlling the degree of pectic breakdown, cell adhesion, cell wall rigidity and permeability characteristics during cooking (Jarvis and Duncan, 1992; van Marle *et al.*, 1994; van Marle *et al.*, 1997).
Determination of a precise function for StTLRP in potato tubers, as well as the identification and roles of the different alleles, is required in order to assign a definite role for StTLRP in regulating potato tuber cooking type.

The efficiency and reliability of using a pooling strategy for studying gene expression has been tested in a number of studies (Agrawal *et al.*, 2002; Kendziorski *et al.*, 2003; Glass *et al.*, 2005) and has shown to be a useful method for reducing the number of microarray hybridizations, however, their experiments did not comprise segregating plant populations. In our study we have shown that a pooling approach in combination with microarray technology and segregating populations is a reliable and efficient way to identify candidate genes associated with a particular trait in plants. However, not all differences in tuber quality are based on differential gene expression. Consequently, one of the major drawbacks using cDNA-microarrays for these types of studies is the inability to discriminate between small allelic variations of genes associated with the trait of interest. Furthermore, the identification of candidate genes by using a dedicated potato cDNA-microarray is limited to the set of genes that are represented on the array, although the dedicated potato array was specifically designed to contain genes involved in the metabolic pathways thought to be associated with tuber quality. In this respect, extending the BSA profiling methodology by including a cDNA-AFLP based approach using the same RNA pools for bulking, increases the number of targeted genes and enhances candidate gene identification significantly (Figure 2). Initial cDNA-AFLP based BSA experiments for a number of potato tuber quality traits in the C x E population have yielded a number of differentially expressed targets representing candidate genes that co-localize with the QTL for the targeted trait on the genetic map (Fernandez del Carmen, unpublished results), validating the potential of this approach. The availability of a genetic map allows the analysis of expression differences as a quantitative trait providing additional information. However, for the sole purpose of identifying novel candidate genes, the availability of a genetic map is not strictly required. Transcript profiling by phenotypic grouping of individuals of a population or different potato varieties or accessions exhibiting a similar quality trait, can result in the identification of high quality candidate genes that can then be confirmed and mapped in populations for which genetic maps are readily available.

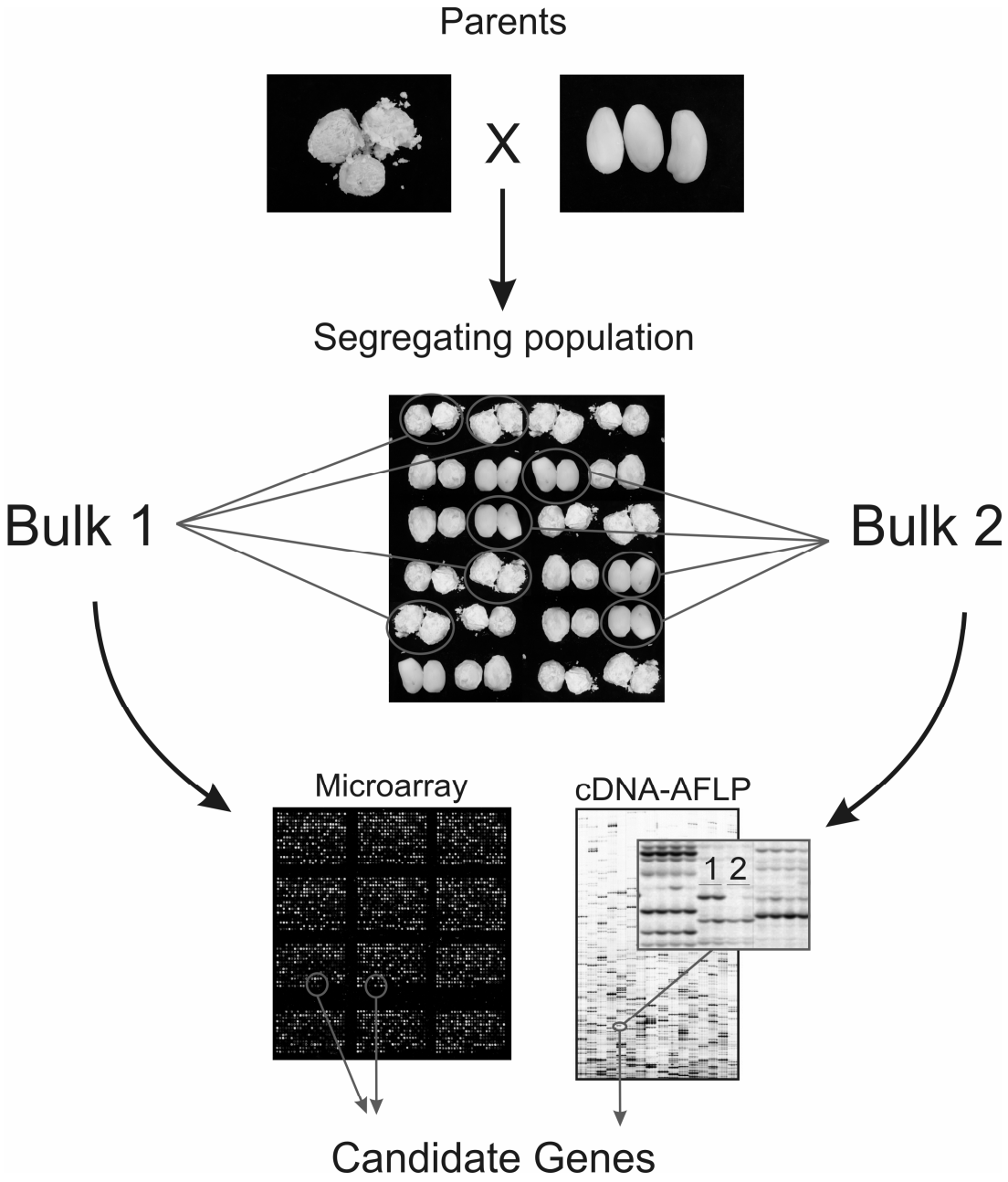

Figure 2; Overview of transcriptomic profiling implementing a BSA approach for the identification of candidate genes using microarray technology or cDNA-AFLP.

Future prospects and concluding remarks

The work described in this thesis shows the successful application of a dedicated potato cDNA-microarray for the study of potato tuber development and quality traits. Based on identified expression differences or profiles, genes may be tentatively associated with a role in a specific developmental process or quality trait. However, linking gene expression profiles to a biological function in complex processes such as potato tuber development and tuber quality remains a difficult task and individual gene function validation studies are still required which are time-consuming and often do not produce distinctive or related

phenotypes, delaying scientific breakthroughs. Transcriptomics has become the first genomics tool that is widely accessible for most molecular labs, and with the rapid development of other large scale genomics tools such as metabolic profiling, the researcher will be able to integrate gene expression and metabolic profiles to efficiently study plant metabolic pathways (Oliver *et al.*, 2002). For example, the presence of health promoting compounds and specific secondary metabolites are becoming more important with regard to plant nutritive values. However, little is known about the metabolic pathways and regulatory genes involved (Verpoorte and Memelink, 2002). Similarly, understanding of metabolic and regulatory pathways involved in determining other economically important tuber quality traits will be vital when attempting to enhance potato tuber quality through either marker assisted breeding or genetic engineering in which the combination of genetics and genomics may prove to be of great value.

With the ongoing development of microarray technology as well as the increasing amount of expressed gene sequence data available to the researcher, the whole-transcriptome analysis for certain crop species is within reach. In potato, the construction of a 44,000 potato oligo array, representing the current unigene set, has recently been completed and allows the study of gene expression on a much larger scale than previously thought possible. In comparison to the dedicated potato tuber life cycle array, described in this thesis, the potential for identifying interesting gene expression profiles during potato tuber development or novel candidate genes associated with specific potato tuber quality traits will be greatly enhanced. Another exciting prospect in the longer term is the completion of the entire potato genome sequence which has recently commenced. The integration of sequence and gene annotation data with large scale transcriptomics and metabolomics studies will greatly advance our abilities to study the potato crop on an entirely new level.

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Summary & Samenvatting

Summary

The potato crop is one of the most important food crops in the world and in order to understand how in potato plants the formation of potato tubers and the determination of different tuber quality traits are regulated, we need to identify and characterize the genes that are involved in regulating these processes. The aim of the research described in this PhD thesis was to implement a relatively new genomic tool (i.e. microarray technology) in an ongoing study to understand the process of potato tuber development and the mechanisms underlying differences in tuber quality traits through the identification and analysis of candidate genes. The induction and formation of a potato tuber from underground stem-like structures, the stolons, follows a developmental program that requires the coordinated regulation of many metabolic pathways. We have developed a dedicated potato cDNA-microarray specifically designed to study the different aspects of the potato tuber life cycle. Genes selected for representation on the dedicated array were retrieved from EST databases using a text mining approach based on functional homology with genes thought to be linked to the process of tuber formation or determining tuber quality traits.

In a large scale *in vivo* tuberization experiment we have analyzed the expression profiles of genes during the transition of a stolon into a tuber and subsequent tuber growth stages using the dedicated potato cDNA-microarray. Expression analysis of eight tuber developmental stages revealed both unique as well as more common gene expression profiles including, up-regulation, down-regulation and transiently up- or down regulation at tuber organogenesis. Based on the observed expression profiles during tuber development, tissue specificity studies and functional homology, promising candidate genes were identified and selected for further analysis. Reverse genetic approaches were implemented to identify functional roles for candidate genes in relation to the process of tuber development or quality traits. One of the most intriguing candidate genes that we identified showed homology to a GA2-oxidase gene involved in the breakdown of bioactive gibberellins (GA) and was named *StGA2ox1*. GA is a plant hormone that has been shown to be an important regulator of tuber formation. When potato plants are induced to tuberize, stolon growth ceases and a reduction in the levels of GA is thought to result in the longitudinal reorientation of the cell micro tubules and micro fibrils, allowing lateral cell expansion and division. *StGA2ox1* is up-regulated during the early stages of potato tuber development prior to visible swelling. Transgenic clones over-expressing *StGA2ox1* exhibit a dwarfed phenotype, reduced stolon length and earlier *in vitro* tuberization. Transgenic plants with reduced expression levels of *StGA2ox1* showed normal plant growth, an altered stolon swelling phenotype and delayed *in vitro* tuberization. Furthermore, tubers of silencing clones contain increased levels of GA_{20}, a precursor of bioactive GAs, indicating altered gibberellin metabolism. Based on these results we propose a role for StGA2ox1 in tuber development through the action of regulating GA levels in the subapical stolon region during early tuberization events.

Another candidate gene that was selected for gene function assessment showed homology to an Aux/IAA protein and was named *StIAA2*. Aux/IAA proteins are short-lived transcription factors that can promote or repress auxin induced gene expression under the

control of the plant hormone auxin. Auxins have long been implicated to play a regulatory role in potato tuber development. However, a precise function for this plant hormone in tuber development has never been established. During the early stages of potato tuber development *StIAA2* transcript levels are dramatically reduced indicating strong transcriptional control. Targeted down-regulation of *StIAA2* transcript levels, through post-transcriptional-gene-silencing (PTGS), results in distinctive phenotypes that include increased plant height, petiole hyponasty and extreme curvature of growing leaf primordia in the shoot apex. Due to lack of a visible potato tuber phenotype in the *StIAA2* transgenic clones, no direct role for StIAA2 in controlling potato tuber development could be assigned. However, additional gene expression analysis of transgenic plants with reduced *StIAA2* transcript levels resulted in the identification of a number of genes with altered expression profiles including another member of the Aux/IAA gene family (*StIAA*), providing new leads in auxin regulated gene expression in potato.

Besides the identification and analysis of candidate genes for a role in potato tuber development, we have used the dedicated cDNA microarray in a novel screening method for the identification of candidate genes related to tuber quality in a segregating diploid potato population (C x E). Isolated mRNA from potato tubers of individuals were pooled based on the observed difference in potato tuber cooking type. Transcript profiling of the contrasting bulks, consisting of individuals with either a mealy or non-mealy texture character, led to the identification of a candidate gene involved in determining potato tuber texture after cooking. The candidate gene exhibits strong homology to a tyrosine and lysine rich protein family and was named *StTLRP*. TLRP proteins are extra cellular cell wall proteins thought to be involved in cross-linking other proteins to the cell wall making them insoluble, thereby potentially modifying cell wall characteristics. It has been well documented that potato tuber cooking type is largely dependent on cell wall characteristics such as rigidity, permeability and level of cell adhesion. Variation in the level of gene expression of the candidate gene was confirmed by quantitative RT-PCR for a subset of individuals of the C x E population. Through the identification of an allelic variant of *StTLRP* and treating the expression levels of *StTLRP* as a quantitative trait, the identified potato *StTLRP* gene was mapped, both as a PCR marker and expression QTL, to the same chromosomal location as the QTL identified for tuber cooking type, strengthening its status as a candidate gene. The combination of transcript profiling with population genetics is often referred to as genetical genomics and is likely to greatly enhance candidate gene identification and subsequent cloning steps. Furthermore, we show that the use of a pooling strategy in a segregating population is a powerful and more cost-effective alternative to hybridizing individuals, and can be easily copied for other potato tuber quality traits or transcript profiling techniques.

Identification of the key regulatory genes for potato tuber development or tuber quality traits will greatly enhance the capabilities for potato tuber crop improvement through marker assisted breeding methods or genetic engineering. The work described in this thesis shows the successful application of a dedicated potato cDNA-microarray for the study of potato tuber development and quality traits and has resulted in the identification of candidate genes that have provided new insights into the complex biology of the potato crop.

Samenvatting

De aardappelplant is één van de meest belangrijke voedselgewassen in de wereld en om te begrijpen hoe in aardappelplanten de vorming van knollen en knolkwaliteitskenmerken worden gereguleerd, moeten we eerst de genen identificeren en karakteriseren die betrokken zijn bij deze processen. Het doel van het onderzoek, zoals beschreven in dit proefschrift, is het begrijpen van de mechanismen die ten grondslag liggen aan het proces van aardappel knolvorming en de verschillende kwaliteitseigenschappen door het identificeren en analyseren van kandidaatgenen m.b.v. een relatief nieuwe genomische techniek (i.e. microarray technologie). De inductie en vorming van aardappelknollen vanuit ondergrondse stengelachtige structuren, de stolonen, verloopt volgens een bepaald ontwikkelingsprogramma wat de gecoördineerde regulatie van vele metabolische routes vereist. Voor dit onderzoek hebben wij een aardappel cDNA-microarray ontwikkeld, specifiek voor de studie naar de verschillende aspecten van de aardappel levenscyclus. Genen die zijn geselecteerd voor representatie op deze array, zijn afkomstig van EST databases die m.b.v. van een 'text-mining' methodiek zijn geselecteerd op basis van functionele homologie met genen waarvan wordt aangenomen dat ze zijn gekoppeld aan het proces van knolvorming of verantwoordelijk zijn voor bepaalde kwaliteitskenmerken.

In een op grote schaal uitgevoerde *in vivo* knolvorming experiment, zijn de expressie-profielen van genen bestudeerd m.b.v. de aardappel cDNA-microarray tijdens de transitie van stolon naar knol en de daaropvolgende groeistadia. De expressie-analyse van acht ontwikkelingsstadia leverde unieke alsook algemeen voorkomende expressieprofielen op, waaronder; up-regulatie, down-regulatie, en transiënte up- en down-regulatie tijdens knol organogenese. Op basis van de waargenomen expressieprofielen tijdens knolvorming, de weefselspecificiteit en functionele homologie, zijn veelbelovende kandidaatgenen geïdentificeerd en geselecteerd voor verdere analyse.

Om de functies van de kandidaatgenen in relatie tot het proces van knolvorming of kwaliteitskenmerken te achterhalen zijn 'reverse genetics' technieken toegepast. Een van de meest intrigerende kandidaatgenen vertoont sterke homologie met leden van de GA2-oxidase genfamilie, betrokken bij de inactivatie van bioactieve gibberellines (GA), en is *StGA2ox1* genoemd. GA is een plantenhormoon waarvan reeds is aangetoond dat het een belangrijke regulator is van knolvorming. Op het moment dat aardappelplanten zijn geïnduceerd om knollen te vormen stopt de stolon groei en wordt verondersteld dat de reductie in het niveau van bioactieve GA's in de stolon resulteert in een longitudinale heroriëntatie van de cel-microtubules en microfibrillen, met als gevolg laterale celexpansie en celdeling. *StGA2ox1* wordt tijdens de vroege stadia van knolvorming up-gereguleerd, nog voordat de eerste zwelling van de stolon zichtbaar wordt. Transgene klonen, die *StGA2ox1* tot over-expressie brengen vertonen een dwergachtig fenotype, gereduceerde stolonlengte en vroegtijdige *in vitro* knolvorming. Transgene planten met gereduceerde expressie van *StGA2ox1* vertonen normale groei maar met een veranderde stolon zwelling fenotype en vertraagde *in vitro* knolvorming. Tevens bevatten de knollen van de silencing klonen verhoogde niveaus van

GA_{20}, een precursor van bioactieve GA's, een indicatie voor een veranderd GA metabolisme. Op basis van deze resultaten veronderstellen wij een rol voor StGA2ox1 tijdens aardappelknolvorming door regulatie van GA's in de subapicale stolon regio tijdens de eerste stadia van knolontwikkeling.

Een tweede kandidaatgen dat is geselecteerd voor gen functiebepaling, vertoont sterke homologie met de Aux/IAA eiwitfamilie en is *StIAA2* genoemd. Aux/IAA eiwitten zijn kort-levende transcriptiefactoren onder controle van het plantenhormoon auxine, die de expressie van auxine-geïnduceerde genen kunnen promoten of verhinderen. Auxines worden al heel lang in verband gebracht met een regulerende rol tijdens knolvorming. Echter, een precieze functie voor dit plantenhormoon in knolontwikkeling is nooit vastgesteld. Tijdens de vroege stadia van knolvorming worden de transcriptieniveaus van *StIAA2* sterk gereduceerd wat een hoge mate van transcriptionele controle weergeeft. De down-regulatie van *StIAA2* transcriptieniveaus d.m.v. post-transcriptional-gene-silencing (PTGS), resulteert in distinctieve fenotypen waaronder, grotere planthoogte, bladstengel hyponastie en extreme kromming van groeiende bladprimordia in de stengelapex. Door het gebrek aan een duidelijk aardappelknol fenotype in de *StIAA2* transgene klonen, kan een directe rol voor StIAA2 in knolontwikkeling niet worden toegewezen. Echter, additionele genexpressie analyse van *StIAA2* transgene planten heeft geresulteerd in de identificatie van een aantal genen met veranderde expressieprofielen waaronder een ander Aux/IAA familielid, *StIAA*. Deze analyses hebben geleid tot nieuwe aanknopingspunten in het onderzoek naar auxine gereguleerde genexpressie in aardappel.

Naast de identificatie en analyse van kandidaat genen betrokken bij aardappel knolvorming, hebben we de aardappel cDNA-microarray ook toegepast in een nieuwe screening methode voor de identificatie van kandidaatgenen gerelateerd aan knol kwaliteitseigenschappen in de segregerende diploïde aardappel populatie (CxE). Geïsoleerde mRNAs afkomstig van aardappelknollen van nakomelingen uit deze populatie werden samengevoegd in bulks op basis van de waargenomen verschillen in kooktype. Transcriptieprofilering van contrasterende bulks, bestaande uit nakomelingen met een kruimige of vastkokende textuur, heeft geleid tot de identificatie van een kandidaatgen dat vermoedelijk betrokken is bij het bepalen van aardappelkooktype. Het kandidaatgen vertoond sterke homologie met de tyrosine and lysine rich protein familie en is *StTLRP* genoemd. TLRP eiwitten zijn extracellulaire celwandeiwitten vermoedelijk betrokken bij het crosslinken van andere eiwitten in celwand waardoor deze onoplosbaar worden wat kan resulteren in veranderde celwandeigenschappen. Het is reeds aangetoond dat aardappelkooktype voor een groot deel afhankelijk is van celwandkenmerken zoals, stevigheid, doorlaatbaarheid en mate van celadhesie. Variatie in het niveau van *StTLRP* expressie is bevestigd voor een deel van de genotypen van de CxE populatie met kwantitatieve RT-PCR. Door de identificatie van een allelische variant van *StTLRP* en het behandelen van *StTLRP* expressie als een kwantitatieve eigenschap, kan het geïdentificeerde *StTLRP* gen zowel als PCR merker als expressie QTL (eQTL) op een genetische kaart worden gepositioneerd op dezelfde chromosomale locatie als de QTL voor kooktype. De combinatie van transcriptie profilering met populatie genetica wordt ook wel 'genetical

genomics' genoemd en zal waarschijnlijk de mogelijkheden voor kandidaatgen-identificatie en klonering sterk vergroten. Tevens hebben we aangetoond dat het gebruik van een 'pooling' strategie in een segregerende populatie een krachtig en een kosteneffectief alternatief is voor hybridisatie van individuele nakomelingen en gemakkelijke toegepast kan worden voor andere knolkwaliteitseigenschappen of transcriptie profileringstechnieken.

De identificatie van de belangrijkste regulatiegenen voor knolontwikkeling en kwaliteitskenmerken zal de mogelijkheden voor verbetering van het aardappelgewas door middel van merker gestuurde veredeling of genetische modificatie sterk vergroten. Het werk beschreven in dit proefschrift laat de succesvolle applicatie zien van een toegewijde cDNA-microarray voor de studie naar knolontwikkeling en kwaliteitskenmerken in aardappel. Het onderzoek heeft geresulteerd in de identificatie van kandidaatgenen die hebben geleid tot nieuwe inzichten in de complexe biologie van het aardappel gewas.

Nawoord

Nawoord

Het is natuurlijk een heerlijk moment om na een lange periode van hard werken deze pagina's te gebruiken om de mensen te bedanken die op wat voor manier dan ook hebben bijgedragen aan de totstandkoming van dit proefschrift danwel hebben gezorgd voor de vaak noodzakelijke ontspanning of afleiding.

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Bjorn

Curriculum vitae

Bjorn Kloosterman werd geboren op 18 september 1976 te Raalte. Na in juni 1992 aan het R.K. MAVO Salland te Raalte zijn MAVO diploma te hebben behaald volgde in 1994 het HAVO diploma aan het Florens Radewijn College te Raalte. In datzelfde jaar begon hij zijn studie laboratoriumtechniek aan de Internationale Hogeschool Larenstein te Velp, met als specialisatierichting plantenbiotechnologie. Zijn stages werden gedaan bij het CSIRO, Divsion of Plant Industry, Canberra, Australië en het voormalige CPRO te Wageningen. In augustus 1998 behaalde hij zijn diploma en in datzelfde jaar startte hij zijn studie Plantenveredeling en Gewasbescherming aan de Wageningen Universiteit. Het afstudeeronderzoek voor deze studie voerde hij uit bij Plant Research International. Hij verkreeg zijn diploma in augustus 2000 met als afstudeerrichting moleculaire plantenveredeling. In april 2001 begon hij als AIO bij het laboratorium voor Plantenveredeling aan de Wageningen Universiteit. Het promotieonderzoek richtte zich op de analyse van gen-expressie tijdens aardappelknolvorming en in relatie tot aardappel-kwaliteitskenmerken, waarvan de resultaten beschreven staan in dit proefschrift. Per 1 mei 2006 is hij in dienst getreden als Post-doc bij het laboratorium voor Plantenveredeling aan de Wageningen Universiteit.

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