Manipulation of the starch composition of Solanum tuberosum L. using Agrobacterium rhizogenes mediated transformation

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CHAPTER 7

SUMMARY AND GENERAL CONCLUSIONS

In this thesis the question is raised whether the starch biosynthesis and/or composition in the potato can be altered by genetic manipulation. In order to manipulate starch metabolism a transformation method is required together with genes which can interfere in this metabolic process.

In Chapter 1 a brief introduction is presented on transformation, with emphasis on Agrobacterium mediated transformation, as well as on starch biosynthesis in higher plants.

Chapter 2 describes the development of a transformation system for potato by combining the use of binary vectors, for the transfer of genes, with the use of A. rhizogenes. The A. rhizogenes strain used was LBA 1334, the binary vector used in this study was pBI121. The latter carried a resistance gene, neomycin phosphotransferase II conferring resistance to kanamycin, and a scorable gene, beta-glucuronidase. Two different approaches were used to obtain hairy roots: one in which stem explants of a diploid and a tetraploid genotype (86.040 and BD86, respectively) were infected with a mixture of A. rhizogenes LBA 1334 and A. tumefaciens LBA 4404 carrying the binary vector pBI121, the other by which the explants were infected with A. rhizogenes LBA 1334 harbouring pBI121. Although the efficiency to obtain hairy roots was equally good with either approach, the percentage of kanamycin resistant hairy roots possessing beta-glucuronidase activity was much higher when the

binary vector was integrated in A. rhizogenes (60% versus 3%). From another diploid potato genotype (HH 578 = PD007) transformed with the latter procedure, hairy root clones were obtained from which nine were further analyzed. From eight of these hairy root clones, shoots were regenerated.

The phenotypical, biochemical and molecular characterization of these regenerated plants is described in Chapter 3. All plants exhibited the typical Ri-phenotype (caused by the TL-DNA of A. rhizogenes), were kanamycin resistant and possessed beta-glucuronidase activity (encoded by vector T-DNA). Some plants also contained agropine synthase (encoded by the TR-DNA of A. rhizogenes). The introduced traits remained stably expressed, even after prolonged culturing under non-selective conditions. No relationship between binary vector T-DNA copy number and the levels of kanamycin resistance and/or beta-glucuronidase expression were found. One striking result was that the transformed plants formed more roots when grown on kanamycin containing medium than on kanamycin free medium. This is in contrast with results obtained with A. tumefaciens transformed plants where the amount of roots formed on selective medium is less than on non-selective medium. Although the majority of the plants was cytologically altered - 58% were tetraploid - the amount of gross cytological aberrations was limited. Molecular analysis showed that the number of integrated vector T-DNA and TL-DNA copies varied from 1 to 5. The number of TR-DNA copies in agropine positive plants was 2. The vector T-DNA sequences were in most cases faithfully integrated, only in one plant a

truncated copy was found. Evidence was found that segregation of the introduced traits can take place after transformation or during regeneration.

Chapter 4 tries to give answers on the questions whether and how the introduced traits are transmitted to an F_1-progeny. Although transformation resulted in reduced male fertility it was possible to obtain seed. Six crosses from a total of 34 different combinations produced berries. Analysis of small F1-populations from four crosses showed that the introduced traits are transmitted to the offspring. One of the most interesting results was that no recombinants for the introduced traits were found. The results, with exception of the rhizogenicity trait in family 8804, are compatible with complete linkage of the marker genes. The molecular data on the Ri-parent plants are in agreement with the results of the genetic analysis.

The results (Chapter 2 and unpublished) show that the transformation efficiency is high with all tested genotypes. In all cases regeneration of shoots from hairy roots is possible within a reasonable time (6 to 10 weeks), although the regeneration percentages may vary (8% to 54%). However, the way by which different genes are integrated into the potato, in apparently the same place in the genome (Chapter 4), renders practical use of this transformation method cumbersome, since getting rid of the undesired hairy root character by recombination will be very difficult. The final goal in this case is to end up with plants which only contain vector T-DNA and nothing else. However, for scientific use, where the presence of Ri-T-

DNA is of no concern, this method is very suitable.

An important enzyme involved in the synthesis of amylose in starch is the granule-bound starch synthase (GBSS). The molecular cloning and characterization of the gene encoding GBSS from a wildtype and an amylose-free potato mutant is described in Chapter 5. Several genomic clones in λ EMBL4 from both potato genotypes were isolated and characterized with the aim to identify the mutation in the amf-1 potato. As in maize about half the number of amylose-free or waxy genotypes are characterized by a structural lesion in the GBSS gene. We attempted to identify such a lesion in the GBSS gene of the amf-1 potato.

It was found that the gene encoding GBSS is a unique gene in potato which is expressed in a number of different tissues involved in reserve or metabolic starch production. Expression is most pronounced in stolons and tubers. No structural difference larger than 50 bp could be found between the GBSS wildtype and amf-1 potato genes. The amount of GBSS RNA present in tubers of the amf-1 mutant appeared to be higher than that in tubers of the wildtype. The absence of amylose and the GBSS protein does not necessarily mean that a mutation in the GBSS gene is involved; a mutation in another gene, although not known in the literature, might achieve a similar phenotype. Studies to elucidate the nature of the amf-1 mutation are in progress. Base substitutions or small changes in the GBSS gene can be traced by sequencing the genes. Another, more conclusive way to investigate whether the mutation lies within the GBSS gene is to perform complementation experiments. Since starch is present in

the columella cells of root tips the presence of amylose can be easily detected. Stained with Lugols solution (IkI) pure amylopectin colours red-brown, whereas mixtures of amylopectin and amylose colour blue. Attemps to complement the mutant with a potato GBSS cDNA construct using the binary vector T-DNA/A. rhizogenes approach gave no positive result. No blue coloured starch was observed in hairy roots induced on amf-1 plants (unpublished results). Since the cDNA might be non-functional experiments with the GBSS genomic clone should give the final answer to the question, whether the mutation is in the GBSS gene. In chapter 6 experiments are described aimed at inhibition of the GBSS activity in potato tubers and thus at affecting the amylose/amylopectine ratio. Heterologous, so called antisense, constructs were introduced by the binary vector/A. rhizogenes transformation procedure described in Chapter 2 into two potato genotypes: H7322 and PD007. Hairy roots which developed on stem segments of both genotypes were stained with Lugols solution (IKI, which stains pure amylopectin reddish-brown and starch with amylose blue) to determine whether reddish-brown columella cells could be obtained, thus suggesting complete inhibition of the GBSS activity. Such cells were indeed found: however, instead of homogeneously staining hairy roots, roots with a pattern of blue and reddish-brown staining cells were obtained. Nevertheless, this indicated that some effect on the GBSS activity was obtained. Plants were regenerated from hairy roots of both potato genotypes and minitubers were induced in vitro. From both genotypes minituber starch was analyzed for its

amylose/amylopectin ratio. This showed that all of the investigated tubers contained amylose. However, as a group the antisense transformants showed a lower average amylose content compared to wildtype transformed plants. A number of PD007 transformants were further characterized for GBSS activity and amount of GBSS protein. The most interesting results from the analysis of these plants were: (1) strong inhibition of the GBSS activity (up to 85%) was not accompanied by a corresponding reduction of the GBSS protein amount and (2) reduction of GBSS activity resulted in only a minor, not proportional, reduction of the amylose content. These results indicate that significant inhibition of the GBSS enzyme activity can be achieved with heterologous antisense constructs, although thus far total inhibition was not observed in the investigated material.

With the cloned potato GBSS gene and the knowledge that antisense GBSS constructs can influence the amylose content of potato starch, powerful tools have become available to manipulate this parameter. Not only can the introduction of the GBSS gene in sense or antisense orientation teach us more about (parts) of the starch biosynthetic pathway, but it might also lead to the production of new potato starches.