Evaluation "Stacking functionally expressed apple genes for durable resistance to apple scab" (WP-034)

Period: June 1, 2006 – June 1, 2010
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Introduction

A major bottle neck in apple production is the susceptibility of almost all elite varieties to apple scab, so that high amounts of pesticides have to be sprayed during the whole season. Classical apple breeding is producing resistant varieties by using resistant wild species. It takes many years of selection and backcrossing in order to remove linked negative traits (linkage drag). The resistance of these new varieties with lower fruit quality, has already been broken at several locations. The alternative is stacking several resistance genes to safe guard resistant apple trees from breaking resistance. But this multiple R-gene approach is a major bottle neck for classical breeding. Therefore, in the IP on Healthy Pip Fruit Chain an alternative GMO (Genetically Modified Organism) approach, called cisgenesis, has been advertised. Cisgenes are genes from the plant itself or from crossable wild species. An advantage is that after cloning of several resistance genes, existing elite apple varieties with high quality fruit can be improved directly by stacking cloned R-genes in one step, without the simultaneous problem of linkage drag.
In the IP project the acceptance and possible exemption of cisgenesis from the EU legislation has been investigated, as well as the cloning of a new broad spectrum resistance gene *V25.*
In this project the molecularly known resistance genes *HcrVf1* and *HcrVf2*, present in resistant cv Santana, have been cloned and tested after transformation into susceptible cv Gala.

Research

PhD student Sameer Joshi worked for 4 years on this technical project in order to come to the first cisgenic resistant plants from the susceptible variety Gala. During the first period research was focused on cloning of the two cisgenic resistance genes *HcrVf1* and *-2* with a long (2kbp) or a short (200bp) own promoter. This was needed in order to compare their expression levels, after transformation, with that of resistant cv Santana. In addition, high expression was induced by using the promoter of the Rubisco gene of apple which is known to be highly expressed. Resistance genes with apple own Rubisco promoter are called "intragenic" because they contain coding parts of different apple genes instead of the situation in transgenes of which at least one functional part is tracing back to non-crossable species.
The above indicated cis- and intragenes have been introduced into susceptible cv Gala by *Agrobacterium* using the pMF1000 vector containing the kanamycin resistance gene for selection and some other genes needed later to remove all helper genes, except the desired *HcrVf* resistance gene. Finally, this removal step has to be made with selected, scab resistant, transformants so that finally marker free cisgenic or intragenic resistant plants have been obtained.

Main results

Plants with the introduced resistance genes in cv Gala have been compared with cv Santana naturally possessing both *HcrVf* resistance genes. Transformation with all 6 gene constructs was successful. Molecular analysis showed frequently insertion of only one or two copies of the resistance gene and that this gene was expressed. The influence of short or long promoter on expression differed in transformed plants with the *HcrVf1* gene but not significantly with *HcrVf2*. A big surprise was that only *HcrVf2* showed resistance in cv Gala. This is clearly indicating that also in cv Santana and other resistant cvs only one *HcrVf* gene is effective.

The transformants were tested with different (a)virulent scab isolates indicating that *HcrVf1* was also not effective in these cases and that resistance spectrum of *HcrVf2* was the same with that of cv Santana. Another observation was that high expression of *HcrVf2* by the Rubisco promoter did not influence resistance spectrum.
The last step was to make transformants marker free and, therefore, cis- or intragenic. Recently, the first marker free plants have been obtained. These plants still have to be further tested. Conclusion is that the circle almost has been closed with obtaining resistant cisgenic apple plants.

Meaning of the results for innovation and sustainable development in agriculture
This project is answering a few important technical questions raised in the IP project. Two known resistance genes have been cloned but after separate insertion into susceptible cv Gala it was shown that only one of them was giving resistance. In such a situation, the breakage of only one effective resistance gene is more quickly expected. The research gives an answer to the important question that not only transgenic but also cisgenic resistant plants are possible. It shows the urgent need of more cloned R-genes to create more durable resistance by gene stacking. At this moment, the earlier mentioned cloned candidate *V25* genes are under investigation in a follow up project. The combination of *HcrVf2* and *V25* is expected to be the basis for making susceptible, but elite varieties, more durable resistant. Such a cisgenic stacking approach needs, including complicated R-gene cloning, 5-10 years and not 50 years as was the case in classical breeding. The obtained cisgenic and resistant cv Gala plants are the first candidates to be used in a cisgenic GMO field trial.

Implications for Metropolitan Agriculture
Sustainable ways of plant production by cisgenesis are crucial for developing durable metropolitan agricultural systems. In the Netherlands, apple is important not only economically for production but also to enjoy the beauty of flowering and fruit bearing orchards by many city dwellers. The intensive fungicide spraying regimes together with the drift of chemicals are bottle necks, especially in (sub)urban areas. Cisgenic gene stacking is a new way to interrupt this practice and to reduce pesticide use. It is expected that apple breeders can make new elite varieties with sustainable resistance to scab and branding them as elite fruit for sale inside and outside the Netherlands.
The cisgenic approach can also be used for other apple diseases and fruit crops and even in all crops. A second example, around cisgenesis, is running in potato against late blight disease. In that case 50% of all pesticides used in the Netherlands are needed to keep potato blight free. A cisgenic solution has also in this case a major impact on making potato cultivation in metropoles more sustainable. So in both cases the triple P approach is valid. It is improving environment, giving added value to the breeder and farmer, and more clean products to the consumer.

Implications for Connecting Values and Agro-Innovations System
This technical project was helping to put cisgenesis on the agenda world wide. Exemption of cisgenesis from the EU GMO directive will bring SME’s and small crops back into the GMO breeding field improving both durability and the agro-innovation system.