CHAPTER 5

General discussion and future outlook

5.1 Summary

Crop improvement for pest resistance has continued to be a challenging task for many plant breeders worldwide. This is partly due to the fact that pest resistance is largely controlled by multiple genes and introgressing them into elite cultivar presents numerous challenges. Banana weevil is not an exception. Breeding of banana is difficult due to sterility, polyploidy and long generation time, and screening large populations of breeding material (hybrids) for weevil resistance is difficult to achieve with conventional breeding techniques. Breeding for such vegetatively propagated long generation crops has been rather by selection of naturally occurring resistance than conventional breeding. Yet, in many cases these selected lines may not have the productivity level as elite varieties. Regardless of the difficulties of generating pest resistance in crops, insect pests continue to destroy crop not only affecting yield in the field but damaging food already in storage.

The most important question facing the future of agriculture is therefore: How can the increased demand for food and other related products be met in the ever increasing world population? In advanced agricultural systems, increased use of fertilizer and pesticides may provide limited benefits, as they are already reaching optimum levels and are damaging to the environment. Similarly, future productivity cannot rely on solely increased irrigation and simply opening up of new lands. These options are also not available to the many resource poor largely subsistence farmers of Africa, Asia and South America. Thus, agricultural productivity and enhanced end-use quality in order to continue supporting humanity will need to exploit the new technologies of modern genetics coupled with environmentally sound cropping systems. Therefore, there has been a recent shift from conventional breeding to biotechnology involving

either molecular markers to pinpoint resistance traits in QTLs or direct engineering of genes from diverse species to crops to enhance resistance to pests and other diseases. One of the most successful control strategies for crop pests has been in recent year the development of *Bt* crops (mainly maize and cotton) that have revolutionalised these agricultural systems. However *Bt* technology is specific to Lepidopteran insects and most Coleopterans insects cannot be controlled in the same way.

At the onset of this PhD study, it was hypothesiszed that protease inhibitors, in particular cysteine protease inhibitors from plants, are potential candidates for the development of banana weevil resistance in banana. To prove this hypothesis, a vacuum infiltration assay was developed in which banana stems were infiltrated with recombinant phytocystatins and then fed to first instar weevil larvae. A first step to prove the correctness of the hypothesis was the finding that the banana weevil mainly employs cysteine proteases in particular cathepsin B and L for protein digestion. A second step of proof was that for the first time a modified *in-vivo* assay could used in which banana weevil larvae were fed stems infiltrated with phytocystatins. It was shown that early developmental rates were significantly reduced by more than 70% compared to the control. However, the presence of multiple forms will present a challenge to the strategy of using a single phytocystatin to target the weevil. Clearly the strategy should consider the use of multiple protease inhibitor forms including both serine type and cysteine types. In this regard, Ortega *et al* (1998) reported higher levels of mortality of larvae of the weevil *Aubeonymus mariaefranciscae* Roudier (Col.: Curculionidae) when fed to diets containing a combination of more than one inhibitor suggesting synergistic toxic effects. Serine proteases are, however, present in mammal digestive systems and would raise considerable food safety concerns when

used in a transgenic crop. Alternatives have to be employed by using either tissue specific or wound inducible promoters instead of constitutive promoter so that the transgene is targeted to a more specific site and time of expression.

A third step of proof was that action of phytocystatins can be improved by site-directed mutagenesis. All protein engineering strategies start with the hypothesis that the target protein has not yet achieved its maximum potential. In this study it has been shown that site-directed mutation was applied to papaya cystatin, 10 mutants showed improvement against papain, 10 against banana weevil gut extracts and 8 against the black maize beetle. This further illustrates the diversity of function and some specificity as some mutants had increased activity in only one of the insects.

By searching for sites for cystatin engineering the evolutionary dynamics of inhibitor-protease interactions and natural selection were also invesitigated in greater detail. This allowed understanding of the evolutionary relationships as well as the diversity in structure and function of phytocystatins. In general, there is a high diversity among phytocystatins. Diversity contains cystartins with functional multiple domains and some cystatins showed up in different taxonomic groups when a phylogenic analysis was carried out. The diversity of evolutionary mechanisms in a single protein family is likely to be due to repeated interactions between these proteins and the continuous pressure to create variation.

By investigating evolutionary relationships the process of positive selection has been proved to occur in phytocystatins. Phytocystatins have amino acid sites that have during evolutionary time undergone positive selection. This study also providesd

some evidence that mutations at these sites adding advantage to the host plant. Such positive selection sites offer the opportunity to modulate phytocystatins for improved activity or specificity.

Overall, this study has ultimately contributed to the advancement of science by providing new findings on the diversity of phytocystatins. It has also provided evolutionary evidence of positive selection in phytocystatins and has shown the importance of functional diversity both in plant defence proteins *vis a vis* pest protease. It has finally shown that cystatins can be improved by changing particular amino acid sites and several novel engineered cystatins have been created that can contribute to developing resistance to the banana weevil and also other Coleopteran insect pests.

5.2 Future outlook

The technology for developing insect-resistant transgenic plants is expanding very rapidly. Such plants have the potential to become in the future a part of the integrated pest management systems both for large commercial plantations but also helping resource poor farmers in Africa. With the development of several transgenic plants expressing *Bt* toxin, which are already on the market, clearly illustrates that gene technologies are a good strategy for developing insect pest resistance that is safe. In this regard phytocystatins have the advantage of very likely less regulatory concern since mammals and humans do not use cysteine proteases in their digestive systems.

More novel engineered cystatin mutants could therefore be created and tested in transgenic plants. In this regard, transgenic tobacco and banana are currently

produced expressing native and engineered phytocystatins to test for their efficiency to control insects. Transgenic approaches also include expression of combinations of different protease inhibitors (serine + cysteine protease inhibitor) or expression of cystatins with multiple mutations to possibly delay resistance in pests. However, the challenge with these proteins will be to develop cystatin variants tailored for the inhibition of specific proteases or sets of proteases. Activity of these target proteases should be efficiently controlled by phytocystatins without interfering with activity of endogenous plant proteases or being degraded by non-target cysteine proteases. Looking at the broader ecosystem level, it may be interesting to use poorly specific inhibitors to increase the number of target proteases, but maybe not an ideal choice as these may inhibit non target proteases unless if their activity can be modulated to make them specific to the targets and less affiants against non-target proteases.