An Overview of Sunflower Disease Research in Australia

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Abstract

Puccinia helianthi, *Alternaria helianthi* and *Sclerotinia sclerotiorum* are the major diseases of sunflower in Australia. Research aimed at developing effective and durable control of these diseases involves the collaborative efforts of researchers from several research organisations which interface with commercial seed companies. The ability to identify and manipulate resistance genes together with an understanding of the dynamics of the pathogen population are fundamental to this research. For sunflower rust, molecular techniques have been developed to identify molecular markers for specific resistance genes. To date, Polymerase Chain Reaction (PCR) -based markers for two rust resistance genes have been developed. Such markers will assist breeders to pyramid resistance genes, and aid in the genetic analysis of undefined germplasm, particularly that used for the differentiation of specific rust pathotypes. At least 20 different rust pathotypes have been identified using differential hosts. However, DNA analysis of different rust pathotypes has revealed a low level of genotypic diversity in the rust population, thereby minimising the potential of DNA fingerprinting for the identification of rust pathotypes. A glasshouse assay for screening germplasm for resistance to *Alternaria* blight has been developed to assist with field-based selection. Recurrent selection using resistant lines identified in glasshouse and field trials is being employed to develop higher levels of resistance. The lack of genetic resistance to *Sclerotinia sclerotiorum* has directed attention towards the use of antifungal proteins for disease control. Crude extracts of proteins that exhibit activity against a number of sunflower pathogens have been identified. Where possible, genes encoding these proteins will be isolated and cloned and transformed into sunflower to provide resistance to multiple diseases.
Key-Words: Resistance, Rust, *Alternaria*, *Sclerotinia*, Molecular markers, Antifungal proteins.

Introduction

The development of the Australian sunflower industry began in the 1960's and expanded rapidly in the late 1970's following the release of the first high yielding hybrids. Since then the size of the production area has fluctuated in accordance with climatic factors and market

forces. In the harsh and somewhat unpredictable Australian environment, sunflower essentially exists as an opportunity crop, sown at any time from spring to late summer depending on rainfall. As a consequence, the area planted to sunflower can vary from 50,000 to 200,000 hectares - a small industry by international standards but nonetheless important to Australias domestic requirements for high quality edible oil.

Apart from environmental constraints, diseases are the most important factors limiting production. Rust, caused by *Puccinia helianthi*, Alternaria blight caused by *Alternaria helianthi* and sclerotinia head and stem rot caused by *Sclerotinia sclerotiorum* are the major diseases of sunflower in Australia. Rust is by far the most important of these and is present in most growing seasons. Since 1983, changes in pathogen virulence have occurred frequently and usually in response to the release of hybrids containing new sources of resistance. The occurrence of *Alternaria* blight and *Sclerotinia* head rot is sporadic, as each disease requires specific combinations of crop growth stage and environmental conditions for full development. Nonetheless, when conditions are favourable for epidemic development, each can cause significant yield losses.

Because of its importance, much of the research conducted in Australia has focussed on rust and has attempted to utilise genetic resistance and strategies associated with the deployment of resistance genes to control the disease. Recently, molecular techniques were incorporated into this program in order to enhance the development of rust resistant germplasm. The development of molecular markers for rust resistance genes and DNA analysis of rust pathotypes are anticipated to improve progress towards the development of more durable control strategies. Breeding for resistance to *Alternaria* blight has taken a traditional approach, with the development of a glasshouse screening assay to complement field-based selection. On the other hand, research into the control of *Sclerotinia* has taken a novel approach, which aims to identify, characterise and clone genes encoding antifungal proteins which ultimately can be used in a generic system of plant transformations.

The goals set by this research require the cooperative efforts of a number of researchers located at different laboratories within different research organisations. Research is coordinated through the Cooperative Research Centre for Tropical Plant Pathology (CRTPP) located at the University of Queensland and which is equipped to conduct molecular research.

Research Objectives

The primary objective of this research is to provide industry with sources of resistance to the major diseases of sunflower, with an emphasis on developing strategies to improve the durability of resistance. Because of the nature of each disease, a different approach has been taken for each.

Collaborators in the research

The Toowoomba laboratory of the Queensland Department of Primary Industries (QDPI) provides the raw materials and complementary traditional research required for the development of molecular markers for rust resistance genes as well as the molecular analysis of the rust pathogen. In addition, this laboratory conducts field and glasshouse screening for the development of germplasm with resistance to *Alternaria* blight. The molecular work required for the development of markers for rust resistance genes is carried out at the Queensland Agricultural Biotechnology Centre (QABC is a section of the QDPI), while

molecular methods for analysing rust pathotypes is underway at the CRCTPP. A generic research program aimed at identifying antifungal proteins is also underway at the CRCTPP. The three commercial seed companies, Pacific Seeds (Zeneca), Agseed Research (Lima Grain) and Pioneer Hi-Bred Australia provide germplasm for molecular marker development, for use as differential hosts for typing rust isolates as well as access to nurseries and field trial sites for the collection of rust pathotypes and other diseases. Commercial companies also provide facilities, equipment and resources for field work when required by other collaborators. Research funds are provided by the Australian sunflower growers through production levies and research grants supplied by the Federal and State Governments.

Rust

Molecular markers for resistance genes

Work at the QABC, is focusing on 1) developing molecular techniques for analysing the sunflower genome and 2) using these techniques to develop molecular markers for sunflower resistance genes.

An analysis of diversity in sunflower using Random Amplified Polymorphic DNA (RAPD) has shown a high degree of polymorphism among different sunflower genotypes. RAPD technology and bulk segregant analysis (Michelmore, Paran and Kesseli, 1991) is being used to identify molecular markers for specific rust resistance genes. Sunflower populations suitable for this type of analysis are being generated by the QDPI and the commercial seed companies. Both public and proprietry germplasm is being targeted. PCR-based markers have been developed for the R1 resistance gene (Lawson et al 1996) and a gene present in the commercial hybrid Advance (unpublished). In both cases flanking markers were identified. These were 4.5 and 26cM either side of the gene in the case of the R1 gene, and 11 and 16 cM either side of the gene in the case of the Advance resistance gene. The accuracy of these markers in detecting the presence of resistance genes in different genotypic backgrounds has been verified using traditional techniques, where resistance genes are differentiated by inoculating plants with specific rust pathotypes. Further work aimed at streamlining and refining the methods used to develop markers is underway and includes improved methods of leaf tissue storage, DNA extraction and a colorimetric test to detect the presence of a specific marker instead of the usual agarose gel method.

This program will continue to identify and develop markers for rust resistance genes and to use markers to assist plant breeders to pyramid resistance genes in commercial hybrids. In this way, it is hoped that rust resistant hybrids can be more easily designed to avoid virulence shifts in the pathogen population. By using resistance genes in combinations, the useful life of individual genes may be prolonged.

Germplasm development

Germplasm from International sources, local wild *Helianthus annuus* and *H. argophyllus* populations and populations derived from random crossings of diverse germplasm are routinely screened for rust resistance using a broad spectrum of pathotypes. Resistant germplasm then enters a cycle of selfing and screening for several generations to fix the resistance. This germplasm is then either released to industry for evaluation in hybrid production and/or returned to a cycle of crossing and re-evaluation.

A number of lines containing pyramids of resistance genes were developed from seven inbred lines, each containing different genes for resistance. The original crosses contained various combinations of either three or four of the resistant inbred sources. Inheritance studies to

determine the number of resistance genes present in both the inbred parents and the pyramided lines are currently underway. Segregating populations used in these studies will be used to develop molecular markers for the genes present.

Traditional and DNA analysis of rust pathotypes

Changes in virulence of the rust pathogen occur frequently, such that rust resistant hybrids have a commercial life expectancy of between 2-5 years. The reasons and mechanisms responsible for these frequent shifts are not clear - the roles of sexual recombination and /or mutation in generating new pathotypes is also largely unknown. Analysis of the rust population using differential hosts has allowed the identification of at least twenty pathotypes. Many of these, we are sure, could be further subdivided given the appropriate differential hosts. At present, twenty-two differential hosts, comprising both public and commercial inbred lines are used to type rust isolates. Few of the resistance genes in these lines have been characterised, and many of the lines contain combinations of resistance genes. Genetic analysis of some of these lines is underway and molecular markers will be developed for specific resistance genes.

Monitoring the rust population in this way is laborious, time consuming and expensive. A rapid and accurate method of pathotype identification using DNA markers is being pursued. Genotypic diversity in sunflower rust populations has been examined using DNA Amplification Fingerprinting (DAF; Caetano-Anolles, Bassam & Gresshoff, 1991), a technique which is similar to RAPD. Fingerprints of isolates of major Australian pathotypes were compared with those of isolates from the Americas, Europe, China and Africa. A greater degree of polymorphism was found between Australian and overseas isolates than was found among Australian isolates. Consequently, Australian isolates exhibited a low level of genotypic diversity compared to the overseas isolates, indicating that theAustralian rust population may have been derived from one or a few introductions. The apparent lack of significant polymorphisms among Australian isolates at this stage, prevents the development of markers for pathotype identification.

An understanding of the mechanisms that drive shifts in virulence may help breeders to pyramid gene combinations that will provide more durable resistance. Under Australian conditions, the asexual stage is able to persist year-round on volunteer hosts and in wild *H. annuus* populations. Mutations may therefore account for much of the variation present. The sexual stage is occassionally observed in the field and its role in generating new pathotypes is currently being investigated. Crosses have been made between isolates of different pathotypes to gain an understanding of the genetics of virulence. A comparison of the recombinants generated from sexual matings, with isolates detected in the field, may give an indication of the relative importance of the sexual cycle. The importance of wild sunflower populations as sources of pathogenic variation is also being investigated.

Alternaria Blight

Resistance to Alternaria blight is quantitative and present at low levels in the sunflower gene pool (Kochman and Kong, 1992). Consequently, molecular teqhniques such as those employed for rust resistance cannot be utilised for germplasm development. Work to date has instead concentrated on developing techniques for identifying resistant germplasm under both glasshouse and field conditions. A glasshouse assay (Kong, Kochman and Brown, 1996) using a selection index based on two components of resistance, mean lesion size and infection frequency, gave a high level of correlation ($r = 0.7$) with field severity ratings for

the same lines (unpublished). This indicates that glasshouse-based selection could be used to speed the development of resistant germplasm. From field data, much of the genetic variation in selected lines was shown to be additive, but heritability estimates based on F_2/F_3 parent-offspring regression indicate that only moderate gains can be expected by selection (b=0.27; unpublished data). The presence of significant genotype x environment interactions further complicates the process of germplasm selection, necessitating the use of field trials across environments and years. Inbred restorer lines with moderate to high levels of resistance relative to the susceptible line B89 were selected from glasshouse and field trials using generated epidemics. These lines have been returned to a recurrent selection program in order to generate higher levels of resistance.

Disease assessment methods for field and glasshouse screening is tedious and time consuming, and subject to operator bias. CRCTPP staff are developing a computer based digital image analysis system that will improve the accuracy and speed of disease assessment. A prototype system evaluated in the glasshouse showed a high level of precision and potential for increasing the speed of rating (Tucker *et al* 1995).

Sclerotinia Stem and Head Rot

Antifungal Proteins

A project is underway at the CRCTPP which aims to identify and characterise plant proteins that have biocidal activity against phytopathogenic fungi. The aim of this research is to produce transgenic crop species using genes that encode specific antifungal proteins. Crop species that either lack resistance in the gene-pool or are difficult to breed for resistance using conventional methods will be targeted. The expression of anti-fungal protein genes will be under the strict control of infection-inducible or wound-inducible plant gene promoters which are also being developed at the CRCTPP.

At present, a large number of crude extracts derived from Australian native plants have been screened. Several of these show good activity against a range of sunflower pathogens (Table 1). On further purification, these extracts are expected to provide a small number of peptides/proteins with specific activity ($< 2\mu g\ mL^{-1}$).

Conclusion

The Australian sunflower industry recognises that disease is a major constraint to production. In particular, rust poses such severe limitatons as to threaten the viability of the entire industry. However, very little rust resistant germplasm obtained from elsewhwhere in the world has been of immediate use in Australia. Commercial sunflower breeders must therefore expend considerable effort in developing rust resistant hybrids. Unfortunately, the high level of variability in the rust population limits the effective life of hybrids to less than five years, thereby diminishing the returns on breeding efforts. Breeding for resistance to both *Alternaria* blight and *Sclerotinia* rot faces the challenge of a sunflower gene pool containing only modest levels of resistance. These are difficult disease problems which will not be solved easily or simply.

The Australian sunflower industry is a small industry with limited funds available for disease research. In order to effectively address this, research efforts of a number of research organisations have been pooled. Solutions to disease problems are sought through cooperation among molecular biologists, plant pathologists and commercial sunflower breeders. The programs utilising this approach are in their early stages, but are making

significant progress. Much of the groundwork for developing markers for rust resistance genes has been completed, and two PCR-based markers for rust resistance genes have already been developed. Germplasm with resistance to *Alternaria* blight has been identified, and the initial work on identifying antifungal proteins for the control of *Sclerotinia* is encouraging. The process of cooperative research seems to be effective, and further progress in each of these areas of research is anticipated.

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Table 1: I.C. 50 values of four crude basic protein extracts against a number of sunflower pathogens

Pathogen	I.C 50 (μg/mL) A.F.P.#1	A.F.P.#2	A.F.P.#3	A.F.P.#4
[1]*Alternaria helianthi* (conidia)	5-25	50	25	5-25
Botrytis cinerea (conidia)	100	>200	50	25
Macrophomina phaseolina ([2]mf)	5-25	100	5-25	5
Phomopsis sp (mf).	5-25	>200	5-25	25
Rhizopus sp. (mf).	>200	>200	>200	200
Sclerotinia minor (mf).	>200	>200	100-200	100
Slerotinia sclerotiorum (ascospores)	200	>200	100	>200
Septoria helianthi (conidia)	5	25	5	5
Verticillium dahliae (conidia)	5-25	>200	5-25	25

1. All cultures were grown on the defined medium as described by Terras et al (1993) except for *A. helianthi*, which was grown on PDA; 2. Mycelial fragments