Molecular Breeding of Transgenic Virus-Immune White Clover (*Trifolium Repens*) Cultivars

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The XX International Grassland Congress took place in Ireland and the UK in June-July 2005. The main congress took place in Dublin from 26 June to 1 July and was followed by post congress satellite workshops in Aberystwyth, Belfast, Cork, Glasgow and Oxford. The meeting was hosted by the Irish Grassland Association and the British Grassland Society.

Proceedings Editor: D. A. McGilloway

Publisher: Wageningen Academic Publishers, The Netherlands

Molecular breeding of transgenic virus-immune white clover (*Trifolium repens*) cultivars

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Keywords: transgenic white clover, alfalfa mosaic virus (AMV), clover yellow vein virus (CYVV), coat protein-mediated virus resistance

Introduction White clover (*T. repens* L.) is a major component of improved pastures throughout the temperate world. It is, however, highly susceptible to virus infection. Alfalfa mosaic virus (AMV), clover yellow vein virus (CYVV) and white clover mosaic virus (WCMV) all contribute to a significant reduction in dry matter yield and persistence of white clover. Sources of natural resistance to AMV in white clover or sexually compatible species are not available. Pathogen-derived resistance strategies, such as the expression of viral coat protein in transgenic plants, thus provides opportunities for the development of virus immune transgenic white clover.

Materials and methods Transgenic white clover plants expressing chimeric AMV coat protein (AMV-CP) genes were generated by *Agrobacterium*-mediated transformation. Selected transformation events were subjected to detailed molecular analysis and virus resistance phenotype. Virus immune events were chosen for transgenic germplasm development.

Results and conclusions The molecular characterisation of over 30 independent transformation events allowed the identification of transgenic genotypes carrying single T-DNA inserts, showing mitotic and meiotic stability of transgene expression (assessed at transcript and protein levels), and AMV immunity evaluated under containment glasshouse and field conditions. Two transformation events in white clover cultivar 'Irrigation' (H1 and H6), with field-immunity to aphid-mediated AMV infection were subjected to multi-site (Hamilton and Howlong, Australia), multi-year (over the 1998-2004 period) and multi-generation (T_0 – T_4) field evaluations and concurrently used for elite transgenic germplasm development. Following top crosses with elite parental breeding lines, diallel crosses of heterozygous offspring plants and identification of AMV-CP homozygous T_2 lines through quantitative PCR-based high-throughput zygocity screening, a breeding nursery with 1,300 transgenic white clover plants was established. A final selection of 37 *syn0* plants derived from both H1 and H6 transformation events was made based on transgene-mediated AMV immunity, non-transgenic CYVV resistance and agronomic characteristics such as plant height, stolon density, internode length, leaf length, flower number, summer growth and survival, autumn vigour and spring vigour. These *syn0* plants were poly-crossed to produce the world's first AMV-immune transgenic white clover cultivar.

In addition, transgenic white clover germplasm was developed in a different genetic background, leading to the selection of 600 T_1 elite white clover plants, following virus infectivity and transgene zygocity screenings. A corresponding breeding nursery was established in Hamilton, Australia in November 2004 for the selection of *syn0* parents for the development of a second transgenic white clover cultivar with transgenic AMV immunity and enhanced non-transgenic CYVV resistance. Furthermore, biosafety research to underpin the release of AMV immune transgenic white clover cultivars was undertaken, such as studies on transgene flow in white clover; development of protocols with highest sensitivity for AMV-CP transgene tracking and tracing in transgenic white clover including a range of downstream products; assessment of AMV-CP allergenicity; assessment of substantive equivalence of AMV CP-transgenic vs non-transgenic white clover at the transcriptome and metabolome level by microarray and FT-MS analysis, respectively.