Spring symposium

Nederlandse Vereniging voor Plantenbiotechnologie en -Weefselkweek
Netherlands society for Plant Biotechnology and Tissue Culture

18 March 2005

WICC-IAC – Wageningen

9:30 Registration with coffee

10:00 Opening by the chairman: **Rob Bogers**

10.10 **Traud Winkelmann** (Universität Hannover)
Somatic embryogenesis in *Cyclamen persicum* - applications for propagation and breeding

10.40 **Krit Raemakers** (WTCC, Wageningen UR)
Transformation of pea (*Pisum sativum* L.) using a cyclic organogenic system

11.10 **Sandra van Bergen** (TNO-Quality of Life, Leiden)
Microspore Culture; "Single Cell Tracking and microsorting"

11.40 **Andy Pereira** (Plant Research International, Wageningen)
Stress tolerance by modification of extracellular matrix

12:10 **Lunch**

12:50 **NVPW Ledenvergadering**

14:00 **Heide Schnabl** (Universität Bonn)
Somatic symmetric and asymmetric hybrids between *Helianthus annuus* and *Helianthus maximiliani*

14.30 **Colin Morgan** (John Innes Centre, Norwich)
Interspecific hybrids in *Brassica*

15.00 **Anton Sonnenberg** (PPO Paddestoelen, Horst)
Mushroom breeding

15.30 **Ronald Zeelen** (TNO-Industrie en Techniek, Delft)
Robotisation of in vitro plant multiplication

16:00 Closing and coffee and tea

Somatic embryogenesis in *Cyclamen persicum* – applications for propagation and breeding

Traud Winkelmann
Institute of Floriculture, Tree Nursery Sciences and Plant Breeding,
University of Hannover, Germany

In the seed propagated species *Cyclamen persicum* there is a demand for vegetative propagation for multiplication of breeding lines as well as for mass propagation. Clonal propagation in cyclamen requires in vitro culture techniques, out of which somatic embryogenesis is the most efficient one. Starting from somatic tissue in ovules, we first induced embryogenic callus on medium containing 2.0 mg/l 2,4-D and 0.8 mg/l 2iP. Applying these growth regulators embryogenic cells were propagated either on solid medium or even more efficiently in liquid culture systems including bioreactors. The transfer to hormone-free medium resulted in differentiation of somatic embryos, which germinated and the plantlets were acclimatized to greenhouse conditions. Beside the high propagation rates, an additional advantage of this regeneration pathway was the stability of the regenerants, which were about 95 - 97 % true-to type. There were genotypic differences observed mainly in the regeneration rate, while callus formation rate was not varying between different genotypes. Detailed genetic analyses revealed a hypothesis for the inheritance of the regeneration ability: Two dominant major genes have been postulated to be responsible for this trait and molecular markers for these are under development. The majority of genotypes were able to develop somatic embryos, so that this system can be used for multiplication of breeding lines where limited numbers of plants are required. On the other hand, the amenability for mass propagation depends on the formation of soft and suspendable callus, which was observed only for a low number of genotypes. With the aim to establish artificial seeds, desiccation and encapsulation of somatic embryos have been studied.

Transformation of pea (*Pisum sativum* L.) using a cyclic organogenic system

Emmanouil N. Tzitzikas, <u>*Krit Raemakers*</u>, Shan Zinhui, Marjan Bergervoet, Jean-Paul Vincken, Richard R.G. Visser.
WTCC Wageningen UR

The regeneration system used for genetic modification of pea, as most other legumes, is based on multiple shoot formation of the axillary buds of the cotyledonary nodes of seed. As a result most of the plants are either escapes or only partly transformed and as a consequence will not pass the transgenes to the next seeds generation.
In the laboratory of plant breeding an alternative regeneration method was develop based on subculture of one node cuttings on thidiazuron (TDZ) supplemented medium. As a result tissue was formed completely covered with buds; so called bud containing callus (BCT).
Transgenic pea plants have been generated after co-cultivation of BCT with *Agrobacterium tumefaciens.* Selection of transgenic tissue was based on visual

identification using luciferase expression followed by physical isolation of luciferase positive. T0 shoots were rooted *in vitro* or during acclimatization in the greenhouse. The transgenic nature of T1 plants was confirmed using luciferase activity.

Single cell tracking and microsorting

Simone de Faria Maraschin, Sandra van Bergen & Mei Wang
TNO-Quality of Life, Leiden

Following abiotic stress to induce barley (*Hordeum vulgare* L.) androgenesis, the development of enlarged microspores in culture was monitored by time-lapse tracking. In total, 11% of the microspores tracked developed into embryo-like structures (type-I pathway), 36% formed multicellular structures (type-II pathway) and 53% of the microspore followed gametophytic divisions, accumulated starch and died in the first days of tracking (type-III pathway). Despite the microspore fate, enlarged microspores showed similar morphologies directly after stress treatment. Ultra structural analysis, however, revealed two morphologically distinct cell types. Cells with a thin intine layer and an undifferentiated cytoplasm after stress treatment were associated with type-I and type-II pathways, whereas the presence of differentiated amyloplasts and a thick intine layer were associated with the type-III pathway. Tracking revealed that the first morphological change associated with embryogenic potential was a star-like morphology which was a transitory stage between uninucleate vacuolated microspore after stress and the initiation of cell division. The difference between type-I and type-II pathways was observed during the time they displayed the star-like morphology. During the transition phase, embryo-like structures in the type-I pathway were always released out of the exine wall at the opposite side of the pollen germ pore, whereas in the type-II pathway multicellular structures were unable to break the exine and to release embryo-like structures. Moreover, by combining viability studies with cell tracking, we show that release of embryo-like structures was preceded by a decrease in viability of the cells positioned at the site of exine wall rupture. These cells were also positively stained by Sytox orange. Thereby, we demonstrate, for the first time, that a position-determined cell death process marks the transition from a mulitcellular structure into a embryo-like structure during barley androgenesis. The results of the cell-tracking were used in the first experiments in the field of microsorting.

Stress tolerance by modification of the extracellular matrix

Asaph Aharoni, Shital Dixit, Aarati Karaba, Rudi Trijatmiko, Nayelli Marsch Martinez, Gert van Arkel & Andy Pereira
Plant Research International, PO Box 16, 6700 AA, Wageningen, The Netherlands

Plants and the environment are separated by an extracellular interface that plays a dual role as a protective barrier, as well as a medium for the exchange of gases, water and nutrients. The primary aerial plant surfaces are covered by a cuticle, acting as the essential permeability barrier towards the atmosphere. It is a heterogeneous layer composed mainly of lipids, namely cutin and intracuticular wax with epicuticular waxes deposited on the surface. We identified an Arabidopsis activation tag gain-of-function mutant *shine* (*shn)* that displayed a brilliant, shiny green leaf surface with increased cuticular wax compared to the leaves of wild type plants. The gene responsible for the phenotype encodes one member of a clade of three proteins of undisclosed function in Arabidopsis, belonging to the plant-specific family of AP2/EREBP transcription factors. Overexpression of all three *SHINE* clade genes conferred a phenotype similar to that of the original *shn* mutant. Biochemically, such plants were altered in wax composition (very long fatty acid derivatives), predominantly downstream of the wax decarbonylation pathway. Total cuticular wax levels were increased 6-fold in *shn* compared to the wild type, mainly due to a 9-fold increase in alkanes that comprised about half of the total waxes in the mutant. Chlorophyll leaching assays and fresh weight loss experiments indicated that overexpression of the *SHN* genes increased cuticle permeability, probably due to changes in its ultra-structure. Likewise, *SHN* gene overexpression altered leaf and petal epidermal cell structure, trichome number and branching as well as the stomatal index. Interestingly, *SHN* overexpressors displayed significant drought tolerance and recovery, probably related to the reduced stomatal density. Expression analysis using promoter-GUS fusions of the *SHN* genes provides evidence for the role of the *SHN* clade in plant protective layers, such as those formed during abscission, dehiscence, wounding, tissue strengthening and the cuticle. They are however not expressed predominantly in the epidermis and thus not the prime regulators of epicuticular wax biosynthesis. We propose that these diverse functions are mediated by regulating metabolism of lipid and/or cell wall components in separation layers of the plant. These functions are conserved between dicots and monocots that show a similar mechanism.

The use and importance of interspecific hybrids in Brassica

Colin Morgan
John Innes Centre, Colney, Norwich, NR4 7UH, UK

There are six species within the Brassica family of which the most important agriculturally within Europe is oilseed rape (*Brassica napus*) and which is an interspecific amphidiploid hybrid between two unknown parents from the diploid species *B. rapa* and *B. oleracea*. While this crop is grown extensively for its oil it is nevertheless a poor crop agronomically and its improvement presents a serious challenge to plant breeders. One reason for this is that there is limited trait variation found within the genetic resources usually available for breeding – a result of oilseed rape's relatively recent origin from only two parents and in the reduction in the number of available alleles resulting from the 'directed selection' of breeding programmes operating within a restricted range of environments. One way of increasing trait variation is through developing 'synthetic' oilseed rape from a range of different diploid Brassica species (especially using the 'wild' forms of *B. oleracea* such as *B. insularis*, *B. macrocarpa* and *B. cretica*). Brassica research at the John Innes Centre has focussed on using these 'synthetic' oilseed rape plants to address a number of problems in oilseed agronomy. Two examples from this research will be described that will illustrate the benefits that can arise from using these novel 'synthetically' derived interspecific hybrid oilseed rape populations. These will describe our approach to addressing the problem of pod shattering in oilseed rape and to improving shoot regeneration and transformation in oilseed rape tissue culture.

Robotisation of in vitro plant multiplication

Ronald Zeelen
TNO-Industrie en Techniek, Delft

Automation of in-vitro plant propagation In vitro plant propagation is very labour-intensive. Since labour rates in western countries are relatively high, production costs are high as well. This has resulted in a movement of propagation activities to low-wage countries. It has become clear however that it is more difficult to control production and product quality. Besides, some companies have experienced problems due to breach of intellectual property rights.

In 2001 a project to automate in-vitro plant propagation was started at TNO. A demonstrator was built following a study of the technical and economic feasibility. The development aims at the completely sterile handling of plant material, which means the equipment must be easy to sterilise. Moreover, to ensure a good propagation process, the fragile tissue must be cut at the right spot with the right equipment. If the benefits of increased production speed outweigh the costs of the machine, then the results will be profitable.

Given the great variation in plant material, a flexible system set-up for the demonstrator was chosen. This includes elements such as a robot capable of moving in six axes, a 3D vision system and specially developed tools for manipulating and dividing the tissue. Currently, the demonstrator is operational and little plants that make up the test crops can now be divided into cuttings.