**BARD Final Scientific Report
Cover Page**

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A functional genomic screen for new plant cytoskeletal proteins and the determination of their role in actin mediated functions and guard cells regulation (feasibility study)

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Feasibility Studies **of up to two years' duration to provide preliminary results for an innovative idea or concept** are sometimes funded by BARD. When such studies result in a request for continued funding based on the results of the Feasibility Study, BARD requires that the Final Scientific report of the feasibility study be included with the proposal in order to allow reviewers to consider the justification of the request. Attached is such a report for the current proposal.

Abstract

The original objectives of the approved proposal were: 1. To construct a YFP fused Arabidopsis cDNA library in a mammalian expression vector. 2. To infect the library into a host fibroblast cell line and to screen for new cytoskeletal associated proteins using an automated microscope. 3. Isolate the new genes. 4. Characterize their role in plants. The project was approved as a feasibility study to allow proof of concept that would entail building the YFP library and picking up a couple of positive clones using the fluorescent screen. We report here on the construction of the YFP library, the development of the automatic microscope, the establishment of the screen and the isolation of positive clones that are plant cDNAs encoding cytoskeleton associated proteins.

The rationale underlying a screen of plant library in fibroblasts is based on the high conservation of the cytoskeleton building blocks, actin and tubulin, between the two kingdoms (80-90% homology at the level of amino acids sequence). In addition, several publications demonstrated the recognition of mammalian cytoskeleton by plant cytoskeletal binding proteins and vice versa.

The major achievements described here are: 1. The development of an automated microscope equipped with fast laser auto-focusing for high magnification and a software controlling 6 dimensions; X,Y position, auto focus, time, color, and the distribution and density of the fields acquired. This system is essential for the high throughput screen. 2. The construction of an extremely competent YFP library efficiently cloned (tens of thousands of clones collected, no empty vectors detected) with all inserts oriented 5'to3'. These parameters render it well representative of the whole transcriptome and efficient in "in-frame" fusion to YFP. 3. The strategy developed for the screen allowing the isolation of individual positive cDNA clones following three rounds of microscopic scans.

The major conclusion accomplished from the work described here is that the concept of using mammalian host cells for fishing new plant cytoskeletal proteins is feasible and that screening system developed is complete for addressing one of the major bottlenecks of the plant cytoskeleton field: the need for high throughput identification of functionally active cytoskeletal proteins.

The new identified plant cytoskeletal proteins isolated in the pilot screen and additional new proteins which will be isolated in a comprehensive screen will shed light on cytoskeletal mediated processes playing a major role in cellular activities such as cell division, morphogenesis, and functioning such as chloroplast positioning, pollen tube and root hair elongation and the movement of guard cells. Therefore, in the long run the screen described here has clear agricultural implications.

The main achievements

The microscope (developed by Zvi Kam)

The screen is conducted on a newly developed microscope system, including computer controlled stage for multi-well plate scanning, fast laser auto-focusing for high magnification imaging, extremely sensitive back-illuminated digital CCD camera (quantum efficiency is >95% throughout the visible and IR wavelengths) and modular controlling software for fully automated operation. The system acquires 80Gbytes of image data per day, and via a clientserver links saves and analyzes the data practically on-line. Recently we implemented a double screen strategy, first at low magnification to identify the positions of all fluorescent cells in each of the 384 wells, then at high magnification at those positions only. This strategy optimizes the throughput to about 40,000 positive cells/day, making a cell-based screen for a completely covered library doable within a short time.

The library (constructed by Einat Sadot and Christopher Staiger). The two groups worked closely to establish the unique library of YFP fused cDNAs of *Arabidopsis* in a retroviral mammalian expression vector. The cDNA library was constructed from two weeks old *Arabidopsis* Columbia seedlings. The library was built using the Clontech Smart cDNA Library Construction Kit (Cat No. K1051-1) that preferentially enriches for full-length cDNAs that are flanked by specific primers containing two different restriction enzymes SfiIA at the 5' and SfiIB at the 3' end. Sequencing of 30 random clones from our first library revealed 30 different full-length cDNAs that contained the 5' UTR, the coding region and the 3' UTR. The expression vector used for the fusion to YFP is the retroviral Clontech pLPCX that was modified as depicted in figure 1. The full length cDNAs were first digested with SfiI and then partial digested with Sau3A that is compatible with BamHI, following this step, cDNA fragments were ligated between SfiI and BamHI upstream to YFP. This strategy allowed us to fuse the cDNAs oriented 5' to 3' to the YFP in three reading frames, giving each and every individual clone the chance to include the YFP in frame.

Figure 1, Schematic representation of the strategy applied for constructing the YFP cDNA library.

The screen (performed by Zvi Kam and Einat Sadot). A pilot screen has been done as follows; bacterial colonies containing the Arabidopsis cDNA clones were individually collected into 60 96- well plates (5 clones per well). DNA was prepared from pools of 2500 clones. Two of the pools were inserted into rat fibroblasts plated in a 384 well plate which was scanned by the microscope.

The saved images were then scored for interesting subcellular localization patterns (Figure 2).

Figure 2. Different subcellular localizations of Arabidopsis cDNAs in Ref52 rat fibroblasts. Rat fibroblasts

(Ref52) were infected with DNA prepared from pools of 5000 different Arabidopsis cDNAs. A collection of different subcellular localization patterns is displayed, demonstrating the validity of this approach.

The isolation of positive clones (performed by Zvi Kam and Einat Sadot). The pool yielding positive clones was sub-divided into pools made from each 96 plate which were

infected and scanned. From the positive 96 well plates we prepared pools from columns and rows as illustrated in figure 3A. Individual positive clones were isolated from the well at the crossing of a positive column and row. The isolated DNAs were re-examined by the microscope by double labeling cytoskeletal markers (for example clone 16 in figure 3B). The full length cDNA was isolated using RTPCR, fused to YFP and re-examined for cytoskeletal localization in the presence or absence of 0.5% Triton X-100, a detergent that washes out unbound cytoplasmic proteins. Importantly, Figure 3C demonstrates that the full length of clone 16 persistently binds actin stress fibers even following the stringent washing with Triton X-100, suggesting a strong and genuine binding (figure 3CB arrows).

Details of cooperation

This screen is entirely novel and based on the unique experience of the partner laboratories and the synergism between them. Chris Staiger specializes in plant actin and actin binding proteins while Zvi Kam specializes in developing sophisticated microscope systems. Einat Sadot links between the two with her previous experience in animal tissue culture and cell biology and present position as a plant cell biologist.

Publications: None