Signal Transduction in Maize and Arabidopsis Mesophyll Protoplasts

Jen Sheen*

Department of Molecular Biology, Massachusetts General Hospital, Department of Genetics, Harvard Medical School, Wellman 11, 50 Blossom Street, Boston, Massachusetts 02114

Plant protoplasts show physiological perceptions and responses to hormones, metabolites, environmental cues, and pathogen-derived elicitors, similar to cell-autonomous responses in intact tissues and plants. The development of defined protoplast transient expression systems for high-throughput screening and systematic characterization of gene functions has greatly contributed to elucidating plant signal transduction pathways, in combination with genetic, genomic, and transgenic approaches.

The availability of mutants, transgenic plants, global gene expression profiles, and genomic sequences has offered invaluable opportunities in understanding organismal plant biology at the cellular and molecular level (Gai et al., 2000; Genome, 2000; Parinov and Sundaresan, 2000; Richmond and Somerville, 2000; Sussman et al., 2000; Walbot, 2000; Zhu and Wang, 2000). Notably, molecular and genetic studies have discovered central components from receptors to transcription factors in diverse plant signal transduction pathways (Bleecker and Kende, 2000; Gray and Estelle, 2000; McCarty and Chory, 2000; Urao et al., 2000; Dangl and Jones, 2001; Inoue et al., 2001; Schroeder et al., 2001; Tena et al., 2001; Zhu, 2001). Still, many missing links exist in the plant transduction pathways from signals to target genes.

Analogous to the mammalian tissue culture lines and transient gene expression assays that are indispensable for the rapid progress in discoveries of signal transduction pathways in multicellular organisms, protoplast transient expression systems using parsley (Petroselinum crispum), maize (Zea mays), carrot (Daucus carota), alfalfa (Medicago sativa), Arabidopsis, and tobacco (Nicotiana tabacum) suspension culture cells have been established. These plant cell lines offer new opportunities to dissect signal transduction pathways involved in UV (Lipphardt et al., 1988), abscisic acid (ABA; Vasil et al., 1989), metabolite (Doelling and Pikaard, 1993), light (Frohnmeyer et al., 1994; Harter et al., 1994), auxin (Liu et al., 1994), defense (Nurnberger et al., 1994), and cell cycle regulation (Evans and Bravo, 1983; Nagata et al., 1992; Ito et al., 2001). Compared with cell culture lines, the use of fresh tissues as protoplast sources offers unique advantages. For example, protoplasts isolated from plant tissues retain their cell identity and differentiated state; they show high transformation efficiency with low maintenance. These freshly isolated protoplasts have proven to be physiological and versatile cell systems for studying a broad spectrum of plant signaling mechanisms underlying phytochrome, clock (Kim et al., 1993), auxin (Abel and Theologis, 1996), gibberellin (GA; Gubler et al., 1999), light, sugar, stress, auxin, hydrogen peroxide (Sheen, 1999; Ten et al., 2001), membrane transport (Maathuis et al., 1997; Bauer et al., 1999; Hamilton et al., 2000; Schroeder et al., 2001), ABA (Uno et al., 2000), cytokinin (Hwang and Sheen, 2001), and cell death (Asai et al., 2000; Bethke and Jones, 2001) controls. With advances made in novel protoplast assays, taking full advantage of the completed plant genome sequences for functional genomic and proteomic analyses of individual plant genes and their products will become a reality.

A BRIEF HISTORY OF PROTOPLAST TRANSIENT EXPRESSION SYSTEMS

Forty years ago, Cocking published the first paper describing a method for the isolation of plant protoplasts (Cocking, 1960). A decade later, the first successful experiment for the introduction of nucleic acid into protoplasts was accomplished by Aoki and Takebe using tobacco mesophyll protoplasts and tobacco mosaic virus RNA (Aoki and Takebe, 1969). Although efficient methods for DNA transfection were not yet available, protoplasts were already a useful tool for investigating cell wall regeneration, cell division, embryogenesis, and differentiation (Kao et al., 1970; Nagata and Takebe, 1970; Takebe, 1971; Vasil and Vasil, 1972), as well as for plant virus research (Zaitlin and Beachy, 1974). Subsequently, protoplasts were isolated from diverse tissues and plants, and shown to retain physiological activities and regulation. For example, freshly isolated meso-
phyll protoplasts perform active photosynthesis and respiration (Edwards et al., 1970; Kanai and Edwards, 1973; Podibelkowska et al., 1975). In barley \((Hordeum vulgare)\) aleurone protoplasts, the endogenous \(\alpha\)-amylase gene is regulated by ABA and GA in parallel to what is observed in seeds (Jacobsen and Beach, 1985). In broadbean \((Vicia faba)\) guard cell protoplasts, \(H^+\) ATPase is activated by blue light (Assmann et al., 1985). Protoplasts also retain cell membrane potentials similar to intact cells and have served as a model system to study membrane transporters. In particular, patch clamping of protoplasts is routinely used to study ion channels and their regulation by light, stress, or hormones (Moran et al., 1984; Schroeder et al., 1984, 2001; Maathuis and Sanders, 1994; Cho and Spalding, 1996; Bauer et al., 2000; Downey et al., 2000; Hamilton et al., 2000). Furthermore, protoplasts are frequently used to analyze calcium signals and regulation in plant cells (Gilroy and Jones, 1992; Trewavas, 1999; Pauly et al., 2000).

The development and improvement of protoplast transformation methods with plasmid DNA by polyethylene glycol (PEG; Krens et al., 1982; Potrykus et al., 1985; Negrottiu et al., 1987), electroporation (Fromm et al., 1985; Nishiguchi et al., 1986; Ou-Lee et al., 1986; Hauptmann et al., 1987; Jones et al., 1989), and microinjection (Hillmer et al., 1992) set the foundation to use protoplasts to study gene regulation and signal transduction in plant cells. The establishment of new, and more economical, convenient and sensitive reporter gene assays for establishment in transient assays. Isolated mesophyll protoplasts usually represent active and homogeneous cell populations (Fig. 1) that are amenable for synchronized pharmacological and biochemical treatments, the analysis of early and transient responses, and photosynthetic gene promoters in maize mesophyll protoplasts (Sheen, 1990).

In the past decade, a few laboratories have employed protoplast transient expression to dissect the functions of cis-elements and trans-factors in many essential processes and signaling pathways. These significant studies have unraveled the control mechanisms of RNA transcription, splicing, transport, and translation in maize and tobacco protoplasts (Callis et al., 1987; Gallie et al., 1987, 1989; Goodall and Filipowicz, 1989; WaiBel and Filipowicz, 1990; Dobbling and Pikaard, 1995; Gallie and Bailey-Serres, 1997); the involvement of VP1, MYB, and bZIP factors in ABA and GA signaling in maize, barley, and Arabidopsis protoplasts (McCarty et al., 1991; Hattori et al., 1992; Kao et al., 1996; Urao et al., 1996; Gubler et al., 1999; Uno et al., 2000); the function of promoters and AUX/IAA proteins and auxin-response factors in auxin signaling in tobacco, pea \((Pisum sativum)\), carrot, and Arabidopsis protoplasts (Ballas et al., 1993; Abel and Theologis, 1994, 1996; Ulmasov et al., 1997a, 1999; Guifoyle et al., 1998), the elicitor- and WRKY factor-mediated transcription regulation in parsley protoplasts (Eulgem et al., 1999), and the important cis-elements and transcription factors for light, phosphate, sugar, and cell cycle regulation in maize, parsley, and tobacco protoplasts (Sheen, 1990, 1993; Frohnmeyer et al., 1994; Graham et al., 1994; Sadka et al., 1994; Ni et al., 1996; Yanagisawa and Sheen, 1998; Ito et al., 2001). Recently, tobacco, maize, potato \((Solanum tuberosum)\), and Arabidopsis protoplast transient expression assays have also been used to study protein stability control (Worley et al., 2000), retrotransposon regulation (Pouet et al., 1991; Takeda et al., 1999), protein targeting and trafficking (Chang et al., 1999; Kleiner et al., 1999; Nimchuk et al., 2000; Jin et al., 2001; Ueda et al., 2001), cell death (Asai et al., 2000), virus movement proteins (Heinlein et al., 1995; McLean et al., 1995; Huang et al., 2000), resistance gene product (Leister and Katagiri, 2000), heat shock proteins and factors (Czarnecka-Verner et al., 2000; Kirschner et al., 2000), protein-protein interactions (Subramaniam et al., 2001), and stress and hormone signaling (Sheen, 1996, 1998; Kovtun et al., 1998, 2000; Hwang and Sheen, 2001; Tena et al., 2001). It is anticipated that more protoplast assays will be developed to dissect a variety of plant signal transduction pathways.

ADVANTAGES AND LIMITATIONS OF MESOPHYLL PROTOPLAST TRANSIENT EXPRESSION SYSTEMS

Much can be learned about gene function and regulation in transient assays. Isolated mesophyll protoplasts usually represent active and homogeneous cell populations (Fig. 1) that are amenable for synchronous pharmacological and biochemical treatments, the analysis of early and transient responses, and
most importantly for DNA transformation. Recent extensive effort in the generation of Arabidopsis knockouts has revealed that the majority of single gene mutants lack overt phenotypes (Bouche and Bouchez, 2001), suggesting the functional redundancy of plant genes under common growth conditions. Protoplast transient expression assays can be used for high-throughput screening of candidate genes even for closely related members of gene families (Sheen, 1996, 1998; Kovtun et al., 2000; Cheng et al., 2001; Tena et al., 2001). Constitutively active and dominant-negative mutants can be rationally created and tested (Tena et al., 2001). The use of epitope and GFP tags enables gene products to be more easily followed and studied in transiently transformed plant cells (Fig. 2; Chiu et al., 1996; Sheen, 1996). Although generating transgenic plants is no longer a formidable task for Arabidopsis (Clough and Bent, 1998), the unpredictable nature of transgene expression and phenotypes still requires major effort, thus limiting the number of genes and constructs that can be analyzed simultaneously. The transient nature of the protoplast assay can also circumvent the difficulty in analyzing genes that cause lethality when deleted or overexpressed in plants. In combination with the increasingly available information on global gene expression patterns (Richmond and Somerville, 2000; Zhu and Wang, 2000), transient assay results can facilitate design of more precise and productive experiments using transgenic and mutant plants.

Because many plant signal transduction pathways are active in mesophyll cells, conserved aspects of plant signaling mechanisms can be established using these cells. The signal transduction pathways found in mesophyll cells can potentially be generalized to other cell types, e.g. root and meristem cells, with the addition of cell type-specific components and/or the use of genes with homologous functions but distinct expression patterns (Hwang and Sheen, 2001). Recent studies have shown that a conserved two-component cytokinin signaling pathway established in mesophyll protoplasts is also active in the root and in shoot meristematic cells (Hwang and Sheen, 2001; Inoue et al., 2001).

Mesophyll protoplasts isolated from fresh leaves have many practical advantages. For example, plant materials are grown from seeds that are genetically stable and more easily stored without subculturating and without needing a sterile tissue culture facility. Non-sterile and differentiated cells are abundant and accessible. Fast and simple procedures have been established to obtain homogeneous, active, and responsive mesophyll protoplasts (Fig. 1) with high transformation efficiency (Fig. 2). The transformation efficiency of Arabidopsis and maize mesophyll protoplasts can reach 90% (Fig. 2) and 75%, respectively (J. Sheen, unpublished data), and cotransfection of multiple plasmids expressing different constructs is very efficient (Abel and Theologis, 1994; Kovtun et al., 1998; Sheen, 1998). Compared with bioilistic transient assays that are less effective, this high level of transformation efficiency enables broader functional analyses of protein products of transgenes in protoplast transient assays. Mesophyll protoplasts can also be isolated from maize and Arabidopsis mutants for cellular and biochemical analysis in transient assays (L. Zhou and J. Sheen, unpublished data; Asai et al., 2000; Uno et al., 2000).

Despite many advantages, conceivable limitations of protoplast transient expression systems also exist. First of all, it is presently not possible to isolate active protoplasts from each plant cell type or from all growth conditions (Power and Chapman, 1985). For example, etiolated true leaves grown in the dark can be obtained from wild-type monocot plants such as maize and barley, but not commonly from dicot plants such as Arabidopsis and tobacco. Currently, etiolated or greening maize leaves provide the best source of mesophyll protoplasts to study synchronous light and sugar regulation of photosynthetic genes (Sheen, 1990, 1991, 1993; Schaeffer and Sheen,

Figure 1. Viability of Arabidopsis mesophyll protoplasts. Arabidopsis leaves were digested with cellulase and macerozyme for 3 h at room temperature. A homogeneous population of mesophyll protoplasts was released and observed under bright field (left) or with a FITC filter to show viable cells stained with a vital dye fluorescein diacetate (right). The purity or viability of the mesophyll protoplasts is usually >95% without gradient purification. Scale bar = 35 μm.

Figure 2. High transfection efficiency of Arabidopsis and maize mesophyll protoplasts. Arabidopsis protoplasts were transiently transformed by the PEG method. Maize protoplasts were transiently transformed by electroporation. A cytosolic GFP marker was used to visualize the transformation efficiency. The mesophyll protoplasts showing only red chlorophyll autofluorescence are untransformed. The transformed cells appear yellow, orange, and/or green. The transformation efficiency is 90% for Arabidopsis protoplasts and 40% for maize protoplasts. Scale bar = 35 μm (Arabidopsis) and 25 μm (maize).
1991, 1992; Jang and Sheen, 1994; Yanagisawa and Sheen, 1998). Establishment of new physiological assays is empirical and can be time-consuming. In the case of transgene overexpression, interpretation of the results must be cautious. Cell walls, plasmodesmata, and cell-cell interactions are lost or interrupted. However, with an optimal supply of nutrients and hormones, Arabidopsis mesophyll protoplasts can actually be used as “stem cells” and the starting point to study cell wall regeneration, cell proliferation, cell-cell communication, embryogenesis, and differentiation (Damm et al., 1989; Masson and Paszkowski, 1992; Wenck and Marton, 1995; Luo and Koop, 1997; Mordhorst et al., 1998).

MISCONCEPTIONS

Although protoplast transient expression assays appear to be simple and straightforward, proficiency requires training, lots of patience, creativity, determination, and a “feeling” for the organism. Because protoplast isolation requires enzymatic removal of the cell wall, there is the mistaken impression that protoplasts are irreversibly wounded and thus are stressed and dying cells. When properly isolated and maintained, protoplasts retain their original biochemical and cellular activities. Based on vital staining with fluorescein diacetate (Larkin, 1976) or Evans blue (Asai et al., 2000), the viability of freshly isolated intact maize and Arabidopsis mesophyll protoplasts is greater than 95% (Fig. 1) for more than 48 h in simple mannitol solution. Most transient assays can be carried out within 12 h after isolation. In fact, Arabidopsis mesophyll protoplasts were used recently as an experimental system to study a specific cell death program induced by fumonisin B1 toxin (Asai et al., 2000). Barley aleurone protoplasts are conducive to study cell death program mediated by reactive oxygen species (Bethke and Jones, 2001). The best evidence against the perception that protoplasts are highly stressed and not suitable for studying signaling is the demonstration that maize and Arabidopsis protoplasts respond to oxidative, heat, and osmotic stress signals and pathogen-derived elicitors as do cells in intact plants (Nurnberger et al., 1994; Sheen, 1996; Kovtun et al., 1998, 2000; Eulgem et al., 1999; Tena et al., 2001). Thus, the “stress” status of mesophyll protoplasts can be quantified using stress-inducible genes. If protoplasts are truly stressed and dying, the general gene expression program is shut down (Asai et al., 2000; J. Sheen, unpublished data). Furthermore, the functionality of plasma membrane proteins in Arabidopsis mesophyll protoplasts has been demonstrated by the detection of cell surface receptor activities for cytokinin and for a peptide elicitor (Hwang and Sheen, 2001; Tena et al., 2001).

For successful and reproducible results, great care should be taken in establishing plant growth conditions (Power and Chapman, 1985; Masson and Paszkowski, 1992), monitoring leaf morphology, age and development, isolating protoplasts, and in testing and comparing various physiological responses between transgenes in protoplasts and endogenous genes in protoplasts and intact plants. Homogeneous populations (>95%) of mesophyll protoplasts are routinely obtained; purity can be easily confirmed by microscopic observation (Fig. 1; Sheen, 1995). Other leaf cell types are generally not released using the established procedure for mesophyll protoplast isolation (Sheen, 1995). Other experimental conditions such as plasmid DNA purity, DNA to protoplast ratio, and protoplast culture density need to be optimized. The method of choice for DNA transfection needs to be tested empirically. For instance, electroporation for maize mesophyll protoplasts and PEG transfection for Arabidopsis mesophyll protoplasts work well (Fig. 2; Sheen, 1990, 1991; Kovtun et al., 2000; Hwang and Sheen, 2001). For each transfection sample, 100 times fewer protoplasts than previously established (10⁶/7) gives optimal gene expression based on the activity of constitutive 35S and ubiquitin promoters (J. Sheen, unpublished data). Depending on the nature of transient expression analysis, one million mesophyll protoplasts could be used for 100 or more transfections and/or assays, a substantial efficiency improvement if plant material is limited. The activities of single cells can also be easily monitored and visualized by vital markers, such as GFP and LUC (Chiu et al., 1996; Sheen, 1996; Kovtun et al., 1998; Yanagisawa and Sheen, 1998; Hwang and Sheen, 2001; Zhu, 2001). Although responses in transient expression assays can be monitored as early as 1 to 2 h after DNA transfection, optimal assay conditions need to be established experimentally. In general, protoplast transient expression analysis is intensive and demanding but enormously rewarding.

DISCOVER AND DISSECT PLANT SIGNAL TRANSDUCTION PATHWAYS

The best demonstration of the fitness of the protoplast transient expression systems for discovering and dissecting plant signal transduction pathways is to provide successful examples (Fig. 3). These studies support the idea that key regulators of plant signaling transduction pathways are conserved in dicots and monocots, and justify the use of model plants such as maize and Arabidopsis. For instance, the discovery of the global sugar repression of photosynthetic gene promoters in maize mesophyll protoplasts is now supported by studies in diverse plant species (Sheen, 1990; Sheen et al., 1999). The proposed role of hexokinase as a sugar sensor based on maize protoplast transient expression analysis is also validated by transgenic plant studies (Jang et al., 1997; Dai et al., 1999) and by the isolation of hexokinase mutants displaying Glc insensitivity in Arabidopsis (Sheen et al., 1999). The negative role of ABA...
Insensitive protein (ABI1) and the redundancy of protein phosphatase 2C (PP2Cs) in ABA signaling revealed by transient expression analysis in maize mesophyll protoplasts (Sheen, 1998) have been confirmed by the isolation of Arabidopsis abi1 null mutants (Gosti et al., 1999) and by studies in a rice protoplast assay (Hagenbeek et al., 2000). Protoplast transient expression analysis has been used for intensive studies of auxin regulated gene expression in diverse plants (Ballas et al., 1993; Abel and Theologis, 1996; Guilfoyle et al., 1998; Ulmasov et al., 1997a, 1997b; Ulmasov et al., 1999). The alternation of IAA17/AXR3 mutant protein stability has also been demonstrated using a protoplast transient assay (Worley et al., 2000). Mesophyll protoplasts have been isolated from Arabidopsis mutants (jar1, etr1, pad4, npr1, acd2, cpr1, and cpr6) and transgenic plants (NahG) to investigate fumonisin B1-induced cell death program that requires ethylene, salicylate, and jasmonate signaling pathways (Asai et al., 2000). The activity of an ABA-regulated reporter gene in a protoplast transient expression assay has been shown to be repressed in the ABA insensitive mutants, abi1 and abi2, but greatly enhanced in the ABA hypersensitive mutant era1 (Uno et al., 2000). Recently, the use of the maize and Arabidopsis mesophyll protoplast transient expression assays has allowed functional analysis of the MAPK signaling cascades involved in oxidative stress, auxin, and defense signaling pathways (Kovtun et al., 1998; Kovtun et al., 2000; Tena et al., 2001). Finally, we have established a quantitative and specific protoplast assay based on cytokinin early response gene transcription (D’Agostino et al., 2000; Hwang and Sheen, 2001). Using this novel system, we have identified a two-component circuitry in Arabidopsis cytokinin signal transduction consisting of four major steps: His protein kinase receptor sensing and signaling, phosphotransmitter nuclear translocation, response regulator-dependent transcription activation, and a negative feedback loop through cytokinin-inducible genes encoding a distinct class of response regulators (Hwang and Sheen, 2001). Analyses of transgenic tissues and plants support the importance of this central signaling pathway in diverse cytokinin responses. This protoplast-based analysis is consistent with genetic characterization of Arabidopsis cytokinin mutants cki1 and cre1 (Kakimoto, 1996; Hwang and Sheen, 2001; Inoue et al., 2001) and with cytokinin-inducible gene regulation in wild-type and transgenic plants (D’Agostino et al., 2000).

The development of various protoplast transient expression assays has broadened the methodology for plant signaling pathway analyses to include biochemical, cellular, genomics, genetic, and transgenic tools. In most cases, discoveries made in protoplasts and conclusions derived from transient expression assays have been supported by transgenic plant studies and/or the isolation and characterization of relevant mutants. It will be possible to use protoplasts isolated from mutants for gene cloning by functional complementation with appropriate transient assays.
FUTURE PERSPECTIVES

Powerful and versatile cell systems using mesophyll protoplasts isolated from fresh leaves of maize and Arabidopsis have been developed. These protoplast transient expression systems show regulated gene expression in response to internal and external signals and allow efficient and penetrating analysis of molecular mechanisms underlying hormone, sugar, stress, and defense signaling (Fig. 3). The use of a combination of tools and diverse resources in the protoplast system offers unprecedented opportunities to answer questions in plant physiology and development. Similar protoplast systems could also be developed using tobacco, barley, wheat (Triticum aestivum), and rice mesophyll protoplasts (J. Sheen, unpublished). The applications of protoplast transient expression systems will continue to contribute to the elucidation of intracellular signaling mechanisms in plants. Observation, imagination, creativity, and commitment are necessary for making discoveries using the protoplast assays. In the model plant Arabidopsis, extensive genetic analyses, genomic sequences, and global gene expression profiles offer a wealth of information to test signal transduction mechanisms in the protoplast transient expression assays (Fig. 3). The conclusions derived from single cell studies can then be readily confirmed using transgenic plants and mutants. It is now possible to develop high-throughput protoplast transient assays for functional genomic and proteomic research, such as to screen for activities and functions of protein kinases, protein phosphatases, receptors, G proteins, and transcription factors (Chory and Wu, 2001; Tena et al., 2001), as well as protein kinase substrates (Cheng et al., 2001). Studies in protoplast systems can provide a framework for whole plant analysis of tissue- or cell type-specific pathways in knockout mutants and transgenic plants.

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LITERATURE CITED


Huttley AK, Baulcombe DC (1989) A wheat α-Amy-2 promoter is regulated by gibberellin in transformed oat aleurone protoplasts. EMBO J 8: 1907–1913


