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Abstract

This monograph presents plant model systems suitable for vital microscopic analysis of excretory function that have been studied by the author during the last 15 years. The approaches to modeling and the screening of similar models that are described may be of interest to the wider ring of biologists working in the fields of cell biology, ecology, medicine, and pharmacology. Without vivisection and fixation, a researcher can observe the processes of secretion and the cellular reactions to exometabolites and can analyze the mechanisms of action. Special models are recommended for studies of cell–cell contacts. Some of the model systems may be used in express-diagnostics for biotechnology, ecological monitoring, and pharmacology instead of animal models.

Preface

Modeling is widely used in biology, in areas such as in genetics, physiology, and pharmacology, where biological, physicochemical, and mathematical models of processes are considered. Biological models are simpler living systems that are suitable for experimental studies. Laboratory animals, plants, and various strains of microorganisms can serve as biological models. To explain the causes of various biological phenomena their mechanisms also can be modeled. Modeling of the life conditions at the levels of individuals, populations, and ecosystems is possible too. Choosing similar models allows the analysis of characteristics, features, and laws of biological processes occurring in real complex organisms. In such models, researchers can reproduce certain conditions that permits the mechanisms of an event or process to be studied. A model process may be demonstrated in artificial systems using genetic disorders or mutants or in natural ones by changes in temperature, light regimes, poisons, etc.

Since the 1970s, there have been attempts to model excretory function at different levels of organization: molecular, subcellular, cellular, tissue, organ, organism, and population levels that described in monographs (Roshchina VD and Roshchina VV 1989, Roshchina VV and Roshchina VD 1993). Currently, there is a need to attract researchers to modeling as one approach to the study of excretory function involving cell donors and cell acceptors of secretion, biosensors that perceive and react to the components of secretion in the form of a physiological response. The aim of this type of modeling is to understand the mechanisms of intercellular signaling and regulation through excretions in the chemical communication of organisms. This monograph is focused on cellular models to study plant excretions in vital conditions by microscopic and spectral methods. The objects should be clearly seen under various microscopes and have intensively colored and/or fluorescent secretory products.

Pushchino, Russia — Victoria V. Roshchina

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Contents

Introduction

The importance of suitable model systems in plant biology is demonstrated by its wide scientific audience (Mandoli and Olmstead 2000). In fundamental studies of plant excretory function, various model systems also may be used. Models are significant not only for understanding fundamental secretory process; the modeling of processes is also one of the experimental approaches to study the mechanisms of intercellular signaling in the chemical communication of organisms (Roshchina VD and Roshchina VV 1989, Roshchina VV and Roshchina VD 1993, 2012).

First of all, model systems are necessary for ecological monitoring to analyze the chemical interactions via excretions between living organisms. The screening of secreting cellular models depends on the aim of the study. The secretory cells of medicinal plants, in which the biosynthesis of physiologically active secondary compounds occurs, seem to be of interest for medical and pharmacological practices. Different cultivation methods (prolonged cultivation, stress factors, and the use of the precursors (predecessors) of the end products biosynthesis) allow their application in the analysis of secretions. Cellular models could be represented mainly as pollen grains, especially in allergology, where pollen excretion induces a reactive immune response in humans.

Model systems may be distinguished as shown in the scheme in Fig. 1. The three main fields of model studies are (1) biochemical investigations (analysis of the composition of secretory products and the chemical nature of the individual components of the secretions, the key biochemical pathways of their synthesis, and the modes of their regulation); (2) direct observation of the secretory process with various quantitative and qualitative methods, from single secreting cells to secretory organs of whole organisms; and (3) genetic manipulation to create mutants with variations in secretory products or secretory organs and enzymes of their synthesis or genetically modifying systems (like green-fluorescing protein included systems) that are used for secretory traffic within a cell. According to the scheme in Fig. 1, researchers can use any suitable model to study changes in composition of secretions, sensitivity of contacting organisms to certain secretions, effects of external factors on secretory processes, etc.

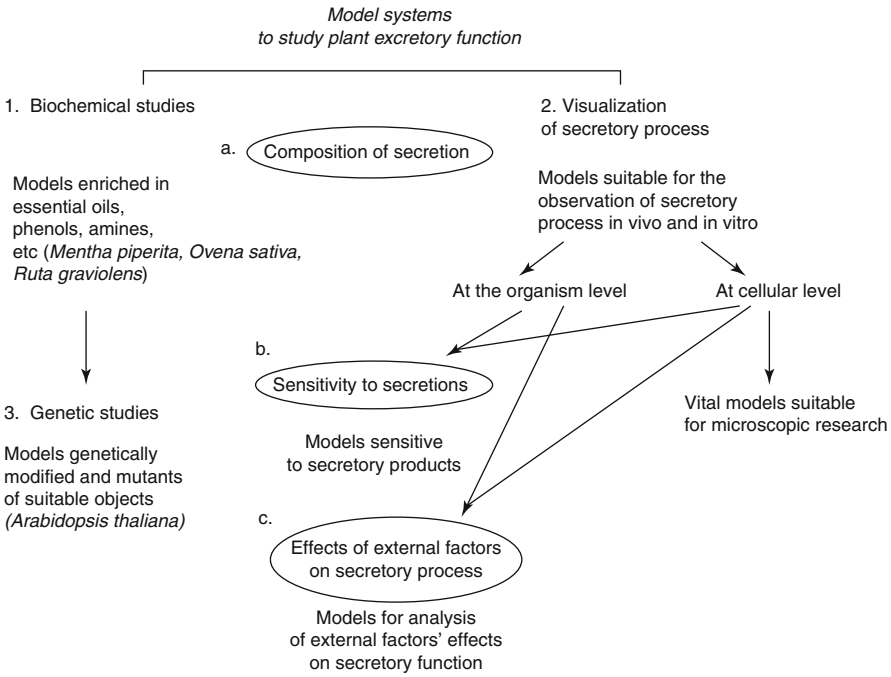

Fig. 1 Modeling in the study of excretory function

Objects characterized as models may be related to whole plants with secretory organs or to individual secretory cells. The modern tendency is to prefer genetic manipulations. *Arabidopsis thaliana* (*The Arabidopsis Book* 2010–2012) is the most multifunctional genetic model for studying secretory organization, including the location of most of the biochemical products, mainly enzymes, in the secretory structures. The wide approach to studies with the *Arabidopsis* model dealt with the biochemical characterization of the components of the secretions (nectars, essential oils, resins, phenolic products, etc.) and the genetics of the secretory products.

Today, the most well-studied model is *Arabidopsis* in terms of the secretions to pollen exine (Dobritsa et al. 2011). The oldest studies with the *Arabidopsis* model began from anatomical research of secretory cells and today they have been followed (a) to the analysis of the biological activity of known secretions or their components on suitable sensitive systems – from microorganisms to plant and animals, and (b) to experiments with the actions of various external factors on the secretory process/internal factors influencing the secretory processes, which are also thought to be an important problem, although not yet studied. A promising example of the use of this model is in the genetic analysis of the structures and composition of nectar and nectaries (Kram et al. 2009). Particularly important are genetic studies that address methods of nectar biosynthesis in the model system of *Arabidopsis thaliana*, where the exploration of the expression of different genes in mutants is convenient (Kram and Carter 2009). Sets of genes encoding components

of the floral nectar called transcriptosomes were found (Kram et al. 2009). Another suitable model for similar analysis is the nectaries of tobacco (*Nicotiana tabacum*) that express genes encoding the synthesis of specific proteins, nectarines; this process is regulated by numerous promoters (Carter and Thornburg, 2000, 2003). Secretory products also are tested in the *Arabidopsis* model, for example, low molecular weight phospholipase A2 regulates cell elongation and shoot gravitropism (Lee et al. 2002). Endocytic and secretory traffic in *Arabidopsis* is often analyzed (Viotti et al. 2010). To analyze the mutants for various purposes, one could study the biochemical pathway of secretory products, for example, in mucilage transport. The epidermal cells of the *Arabidopsis* seed coat undergo a complex differentiation process in which there is sequential synthesis of pectinaceous mucilage followed by secondary cell wall production (Arsovski et al. 2010). The release of mucilage from secretory cells is a model to study the processes and regulation of cell wall production and modification during plant development. In addition, the polar secretion of pectin during mucilage production also makes this system a good model with which to dissect the mechanisms of targeted secretion in plants, and is used in the T.L. Western laboratory (Harpaz-Saad et al. 2011).

The biochemical pathways of essential oils (Dudareva and Pichersky 2006; Gershenzon and Dudareva 2007) and genetic engineering of medically valuable monoterpenes are studied in the leaves and flowers of *Mentha piperita* as a model (Turner and Croteau 2004; Wildung and Croteau 2005; Turner et al. 2012). Similar models for analyses of the composition of secretions, for example, the assortment and variability of essential oils in the flowers and leaves of *Hypericum perforatum* L. are valuable (Radusiene et al. 2005). Tissue root and leaf cultures inoculated by various microbial agents in collections of 30 species of plants belonging to 17 families are also used as such models in the studies of secretory alkaloids; for example, *Peganum harmala* L. for analysis of β-carbolinic alkaloids (Berlin et al. 1994; Kuzovkina et al. 2004).

The consideration of secretory cells as individual systems led to particular attention on the problem (Wagner 1991) and to the elaboration of methods for the isolation of secretory structures that could be applied for chemical analysis (Gershenzon et al. 1987a, b; Yerger et al. 1992; Goodger et al. 2010). This approach to the modeling of plant excretory function based on isolated secretory cells (Gershenzon et al. 1987a, b) allows the direct study of the biosynthesis of plant natural products in isolated secretory cells. In particular, such model may be intact subdermal secretory cavities (relatively large and rich in essential oils) from *Eucalyptus* leaves with cavities (Goodger et al. 2010). Isolated secretory root hairs are also good models for the study of the biosynthesis of lipid resorcinols and benzoquinones (Dayan et al. 2007). A new method that combined hollow fiber liquid-phase microextraction with in situ derivatization combined with gas chromatography–mass spectrometry was also applied to the analysis of the root exudate of *Capsicum annuum* L. (Sun and Wang 2013).

Special attention is paid to sensitive unicellular models used for the analysis of effects of exometabolites, independently on the plant or microorganism is studied. For example, compounds evoked from cultures of yeast *Saccharomyces cerevisiae*

(Raghuraman and Brewer 2010; Oleskin et al. 2013) may influence other organisms. In this aspect, the invasion and defense mechanisms related to the truncation of chitinases from *Arabidopsis* by secreted fungal proteases can be analyzed (Naumann and Price 2012). In earlier publications, pollen grains were chosen as suitable models for the analysis of cell wall and exine formation (Sheldon and Dickinson 1986; Takahashi and Skvarla 1991) or male sterility (van der Veen and Wirtz 1968).

Researchers also need to understand the complex mechanisms of the effects of exometabolites in biocenosis and the cellular mechanisms of interspecies relations with respect to the problem of invasive plants in various ecosystems (see the book edited by Kohli et al. 2009) or allelopathy (Inderjit et al. 1999; Narwal et al. 1999a, b). Among different natural models, there are mathematical models, for instance, for the study of nectar secretion in animal-pollinated plants (Sakai, 1993) as well as for the analysis of the biological response to allelochemicals (An et al. 1993).

The toxicity of various products excreted by plants seems to be testable with various models such as two mammalian cell lines, Neuro 2A and Vero, in the green alga *Chlamydomonas reinhardtii* and the bacteria *Vibrio fischeri* (Perreault et al. 2012). In addition, this is possible to do on animal unicellular models that are easily cultured in the laboratory, in particular, Ciliates, e.g., *Tetrahymena* and *Paramecium* (Beisson et al. 2010). The unicellular model system is also useful for investigations of the multigenerational effects of hormonal imprinting (Kőhidai et al. 2012; Csaba 2012), and for observation of chemotaxis and cellular differentiation, such as in *Chlamydomonas* cells (Ermilova 2013). Plant excretions and individual components of secretions may also be tested in animal multicellular models, such as Planaria serving as a model system analogous (similar with) Mammalia because of specific behavioral patterns that are analogous to mammalian stereotypes in response to drugs acting on acetylcholine or dopamine transmission (Buttarelli et al. 2000). Planaria as a model organism is uniquely poised to be used to investigate the mechanisms of tissue regeneration, stem cell regulation, tissue turnover, pharmacological action of diverse drugs, cancer, and aging (Oviedo et al. 2008). It is also amenable to molecular genetic techniques aimed at understanding complex biological tasks.

Various models may be used for practical application. Of particular interest is the topic of plant models for the study of mechanisms in plant and human pathogenesis because plants, microorganisms, and humans have common means of secretion and genetic regulation (Lugtenberg et al. 2002; Guttman 2004). All type of cells share many virulence factors, such as extracellular polysaccharides and some type-secreted effectors, and have common virulence mechanisms. Common principles and mechanisms play roles in the interactions of microbial pathogens, biofertilizers, phytostimulators, rhizoremediators, and biocontrol agents with plants (Lugtenberg et al. 2002). Special emphasis is given to colonization, phase variation, two-component systems, quorum sensing, complex regulation of the syntheses of extracellular enzymes and secondary metabolites, etc. The study of the composition of secretions and their effects in model systems, such as plant–nematode, provides information that is useful for phytopathologists (Vanholme et al. 2004).

Seeds from some economic plants released volatile metabolites that serve as protector compounds against parasites are of especial interest in phytopathology for modeling plant-parasite relations (Roshchina and Roshchina 1993, 2012), but it should keep in mind that simultaneously the excretions are the sole carbon and energy source for some bacteria (Sidorenko and Buzoleva 2012). Relations "plant-insect" may be studied on special plant models which floral scent with the compounds attracts insects-pollinators that is necessary for succesful pollination in plant fertilization (Dobson and Heidi 2006).

The recent tendency to understand natural events in comparison with technology includes two criteria proposed to characterize the diverse relations between nanotechnology and Nature (Schiemann 2005). Assuming that nature is not produced by human action, the first criteria endorses the difference between natural and artificial objects in nanotechnology and the second criteria allows the discussion of potential nanotechnological modifications in nature. The selection of vital models sensitive to small concentrations of certain chemicals may serve as part of a similar technology.

In this book, widely known models are not described, referring readers to special literature. Instead we concentrated on cellular models (not much known) that can be used in vital regimes without fixation and vivisection. The focus of this book is on new cellular models analyzed with in vivo microscopy that permit estimation of the accumulation and release of secretions and their biological effects, including signaling and contacts with other cells.

Chapter 1
Approaches to Choice of Model Systems for Microscopic Studies

Modeling with secretions and receipting systems usually includes the analysis of such reactions as composition of the secretions and their spectral characteristics (absorbance and fluorescence), the germination, and the growth and development. In this case, the donor model of the secretion and the acceptor model of the secretions should be distinguished. Modeling processes involve donor cell secretion (model system 1) and acceptor cells-biosensors (model system 2) that perceive and react to the components of this secretion in the form of a physiological response, which is one of the experimental approaches to study the mechanisms of intercellular signaling in the chemical communication of organisms (Fig. 1.1). Donors may be considered not only as plants because excretions of other organisms enriched in similar secretory products, for example, known neurotransmitters acetylcholine and biogenic amines as well as related enzymes of their synthesis and catabolism, are observed in animals and microorganisms (Roshchina 2010). Plant cells also serve as acceptor models of the external chemicals independently on the organism which adds the secretion (microorganism, animal, or other plant species). As shown in Fig. 1.1, the contact of models 1 and 2 in turn may serve as model 3 when both contacted models changed their parameters in the form of physiological replies.

To choose model systems suitable for microscopic observation is a necessary step in the studies. The objects used as intact model system can be well seen under various microscopes, having intensively colored or fluorescent secretory products. In many cases, they fast germinated or developed during the short time of visualization. The researcher also observes the localization of secretory products and their accumulation in secretory structures on the different stages of plant development as well as express identification of the prevailing component. The technique for analysis may be suitable for the investigation of mechanisms when one cell (a donor cell) releases a molecule that is received by another cell (an acceptor cell) belonging to a different species.

The success of such investigations requires models, mainly pigmented and/or fluorescent cells suitable for visual manipulation under the microscope and, in some

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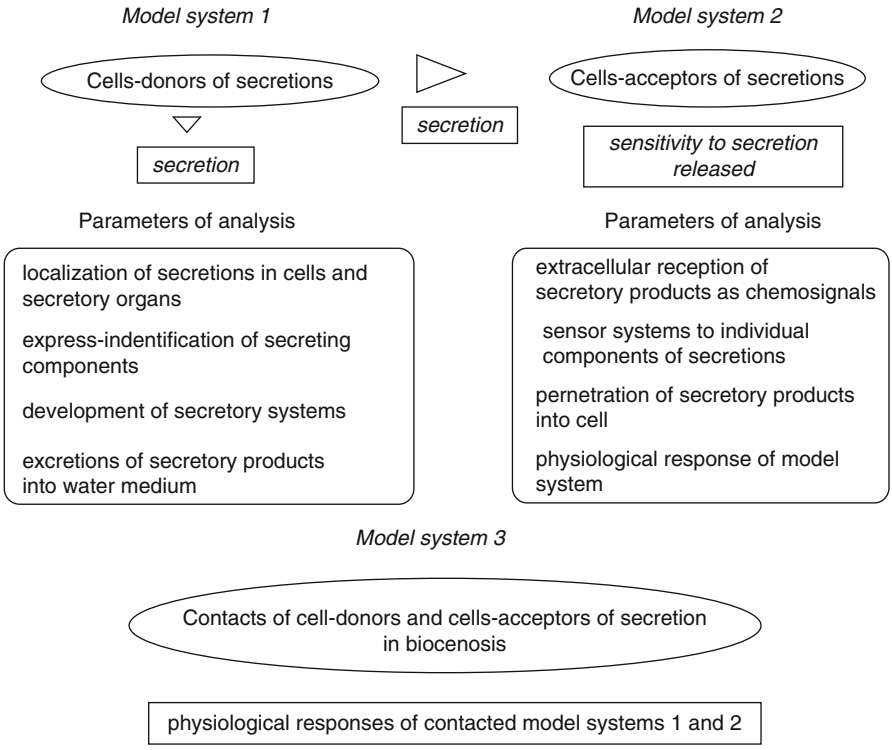

Fig. 1.1 Model systems which could be used for microspectral analysis

cases, measurable as the absorbance or fluorescence parameters. As a whole, the search is concerned mainly with biologically (allelopathically) active plant species which also possess medicinal features. Multicellular or unicellular objects may be chosen as models depending on the purpose.

It is possible to observe cellular models by microscopic methods: (a) the transport of the compounds analyzed into an acceptor cell selected as a model (biosensor), (b) changes in the autofluorescence or color of model cells, (c) the subcellular location of secretion in models, (d) interaction of exometabolites from a donor cell with certain compartments of the acceptor cell, and (e) changes in growth and development of the acceptor cell as an integral response of the biosensor to secretions from a donor cell.

The success of such investigations requires models, mainly pigmented and/or fluorescent cells suitable for visual manipulation under the microscope and, in some cases, measurable as the absorbance or fluorescence parameters.

Cellular models are possible to be observed by microscopic methods: (a) the transport of the compounds analyzed into an acceptor cell selected as a model (biosensor), (b) changes in the autofluorescence or color of cells, and (c) the subcellular location where the chemical acted within the acceptor cell (model) and interacted with certain compartments.

The use of the cellular models is possible to observe by microscopic methods for the analysis of cell–cell contacts. In this case, one could see the changes in the state of both the donor cell of the secretion and the acceptor cell of the secretion. Among the processes tested may be the following: (a) the transport of the compounds analyzed into an acceptor cell selected as a model (biosensor), (b) changes in the autofluorescence or color of donor cells or acceptors of the secretion, and (c) the subcellular location where the chemical acted within the acceptor cell (model) and interacted with certain compartments. Images of contacting surfaces with secretions may be observed by usual microscopy, stereomicroscopy, and various modifications of luminescence microscopy, including laser-scanning confocal microscopy and microspectrofluorimetry.

In this chapter, the various approaches of secretory process testing with microspectral and usual spectral techniques that could be used for various model systems are considered.

1.1 Color and Absorbance in Analysis

Images of contacting surfaces with secretions may be observed by usual microscopy, stereomicroscopy, and various modifications of luminescence microscopy, including laser-scanning confocal microscopy and microspectrofluorimetry. In some cases, the choice of the objects recommended as a model could be connected with the possibility to receive spectral data with microspectroscopy or microspectrofluorimetry as well as by using special apparatuses related to laser-scanning confocal microscopy. Visual observation and spectral measuring of colored samples may occur under the usual microscope or stereomicroscope and various spectrophotometers (Table 1.1). The luminescence technique includes various luminescence microscopes and their modifications such as microspectrofluorimeters (combination of the luminescence microscope with a multiphotometer for the emission spectra registration) and laser-scanning confocal microscopes (a technique which permits to do optical slices of the object and to register the emission spectra from different parts of the sample). Optical coherent images (registered by optical coherence microscopy) could also be received for secretory structures impregnated with calcium or siliceous salts, in particular idioblasts including oxalates of calcium or tips of stinging emergences (Kutis et al. 2005; Roshchina et al. 2007a).

1.1.1 Usual Microscopy and Stereomicroscopy

Usual microscopy is useful when the samples contain pigmented secretions well seen, as can be observed in particular for the leaf oil ducts and secretory cells of *Hypericum perforatum* (Fig. 1.2). The main pigment of oil ducts colored in red is

Table 1.1 Technique for the study of colored plant samples

Apparatus	Aim of the study	Material for the study	Advantages of the methods
Usual microscope with transmitting light	Visual and photographic analysis of the secretion of intact secretory cells	All parts of plants	Simplicity and noninvasive observation
Stereomicroscope	Visual volume and photographic analysis of intact secretory cells	All parts of plants	Simplicity and noninvasive observation
Microspectrophotometer for the spectrum recorder	Detailed spectral analysis of the secretions of intact and fixed secretory cells	All thin parts of plants	Noninvasive observation and receiving of spectral quantitative and qualitative characteristics of the sample
Usual spectrophotometer with different cuvettes, glasses, and glass cameras	Spectral analysis of the absorbance of intact secretory cells	All parts of plants	Noninvasive observation and receiving of spectral quantitative and qualitative characteristics of the sample

known to be anthraquinone hypericin (Brockmann et al. 1950) and used as medical drug (Karioti and Bilia 2010). The absorbance spectrum of hypericin has maxima at 590 and 595 nm (Roshchina 2008). Colorless secretory cells do not include the pigment. Other examples may be the leaf phenol-containing glands of *Lysimachia nummularia* (Fig. 1.3). Some secretory cells of the genus *Lysimachia* include both phenols and their quinones (Fig. 1.4), for example, benzodilactones and quinones in glands of *Lysimachia fordiana* (Huang et al. 2009).

In practice, the accumulation of vacuolar pigments is analyzed. In vacuoles, anthocyanins are usually concentrated (Conn et al. 2010), although instead of these flavonoids, two major types of betalains, the red-purple betacyanins and the yellow-orange betaxanthins, are met (Harris et al. 2012). The color of many flower petals and sometimes non-generative parts of plants permits the observer to see internal secretory structures as vacuoles simply under the usual microscope with water or oil immersion.

Stereomicroscopy is used mainly for multicellular objects to be seen in bright light. Modern stereomicroscopes are equipped with a zoom lens system or a rotating drum (containing Galilean telescopes) that are utilized to increase and decrease overall magnification (achieved by objective and eyepiece magnifications, plus that contributed by any intermediate or external auxiliary magnifying lens systems). Stereomicroscopes are used for the observation of secretory structures and the natural secretions on the surface of allelopathic plants, in particular essential