Signalomics: Diversity and Methods of Analysis of Systemic Signals in Plants

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Contents

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Abstract

We provide a brief definition and history of signals, pointing out how differences in body plan between plants and animals require fundamentally different signaling mechanisms, and then list the diversity of chemical and physical signals along with their pathways of transmission, providing details on molecular signals and focusing on the phloem and xylem as being the main conduits for (rapid) systemic signaling. The two major electrical (action potentials and variation potentials) as well as hydraulic signals are then described. The latter part of the chapter deals with methods of analysis of molecular signals, including accessing the phloem and identifying the array of gene products transported therein. A description is provided of the modern methods used in metabolomics and phenotyping to analyze the metabolic consequences of signal action. Conventional techniques for analyzing electrical and hydraulic signals and their ionic components using electrodes are then furnished. Finally we describe novel techniques

developed recently in the animal field using fluorescence to monitor real-time changes in membrane potential, which could be adapted for plants to open up new vistas in our understanding of electrical signals in plants.

Abbreviations

ABA(-GE)	Abscisic acid-(glucose-ester conjugate)
CBL	Calcineurin B-like
CIPK	CBL-interacting protein kinase
CML	Calmodulin/calmodulin-like protein
eATP	Extracellular ATP
DAG	Diacylglycerol
FRET	Fluorescence (or Förster) resonance energy transfer
FTIR	Fourier transform infrared spectroscopy
GC-MS	Gas chromatography-mass spectrometry
PLAFP	Phloem lipid-associated family protein
PI	Protease inhibitor
PIIF	Protease inhibitor-inducing factor
RALF	Rapid alkalinization factor
ROS	Reactive oxygen species
SE	Sieve element
SP	System potential
VP	Variation potential
VSFP	Voltage-sensitive fluorescent protein
VOCs	Volatile organic compounds
VT	Voltage transient

Introduction

Signals are packets of biological information generated in one location and transmitted elsewhere, frequently in response to external (environmental) stimuli. After a signal has been transmitted, it acts as an internal stimulus to evoke downstream responses. Hence signals and stimuli are the fundamental units of biological communication (Davies 2004). Signals vary in identity, velocity, informational capacity, and locality (site of generation, transmission, and action).

Over 220 years ago, electrical signals were the only ones known in both plants and animals (Galvani 1791). In the middle of the nineteenth century, chemical signals were discovered in animals, and the field of endocrinology developed (Berthold 1849). Now animals were "superior," insofar as they had both electrical and chemical signals, whereas plants had just electrical signals. It was about 30 years later before Charles Darwin (1881) furnished the first evidence in plants for the existence of a diffusible chemical, which could cause plant cell enlargement. For some reason the discovery of chemical signals (hormones) in plants led to the demise of a role for electrical signals despite the previous 100+ years of research showing their existence in plants (references in Stern 1924). After Darwin (1881), the next attempt to assign signal transmission in plants to a chemical was proposed 35 years later by Ricca (1916) to explain the propagation of the signal that causes pulvinus movements of leaflets in *Mimosa pudica*. While the nature of such molecules remained unknown for a long time, numerous molecules have now proven their ability to carry information a long distance in plants.

Higher animals are heterotrophic and thus need to be motile to locate food and partners for reproduction, while food and gas exchange takes place through interior tubes. This demands that they have a near cylindrical body plan to contain the tubes and appendages for motility, and so they have a very low surface area to volume ratio. In marked contrast, plants are autotrophic, rely on animals or the wind to aid in reproduction, and thus have no need to be motile. Consequently, plants are not able to escape from their abiotic (cold, heat, etc.) or their biotic (herbivores, insects) environment (Maleck and Dietrich 1999). Furthermore their food and gas exchange takes place through external surfaces (leaves for light and CO_2, roots for ions and water), thereby giving them a high surface area to volume ratio with many of their cells in direct contact with the environment (Hallé 1999; Vian et al. 2007). It

Table 1 Signaling distance/pathway in plants

Transmission	Examples	References
Within the cell	Calcium	Dodd et al. (2010) and Stael et al. (2012)
Diffusion, secretion, cytoplasmic streaming	DAG	Jeannette et al. (2010) and Canonne et al. (2011)
	Protons	Kader and Lindberg (2010)
	Kinases	Tena et al. (2011)
	H_2O_2 waves	Vestergaard et al. (2012)
Between cells	IP_3	Gillaspy (2011)
(Apoplasm and/or symplasm)	Auxin, ABA	Blakeslee et al. (2005) and Umezawa et al. (2010)
	ROS	Mittler et al. (2011)
	Oligosaccharides	Shibuya and Nimami (2001)
	Peptides	Murphy et al. (2012)
Between organs	Molecules in xylem	Atkins and Smith (2007) and Krishnan et al. (2011)
Phloem and/or xylem	Molecules in phloem	Dinant and Suárez-López (2012)
	Hydraulic signals	Malone (1993)
	Electrical signals	Davies (2004) and Yan et al. (2009)
Between plants	Phenolic compounds	Treutter (2006) and Cesco et al. (2012)
Airborne transmission, mycorrhiza	Jasmonate and methyl jasmonate	Avanci et al. (2010)
	Ethylene	Wilkinson and Davies (2010)
	Mycorrhiza	Song et al. (2010)
Between generations	DNA methylation	Migicovsky and Kovalchuk (2013)
Gene modification	Histone modification	Bilichak et al. (2012)
	Gene silencing	Qutob et al. (2013)

becomes then tremendously important for plants to sense even minute changes in climatic or mineral resources in the environment and adapt their growth (Läuchli and Grattan 2007) or flower production (Suárez-López 2005) accordingly and also to promote defense strategies. Indeed, environmental factors such as nitrate availability (Forde 2002; Liu et al. 2009), wind (Anten et al. 2010), drought (Hetherington 1998; Jia and Zhang 2008), flooding (Dat et al. 2004), predator attacks, or infection (Heil and Ton 2008; Parker 2009) induce systemic responses in plants that generally result in a reduction in growth and yield.

Plants must, therefore, possess systems to exchange information throughout the entire plant to ensure the coordination of plant development and defense. The systems for transmitting this information are complex and involve multiple components, which are far from being understood. Evidence strongly suggests that information exchange relies on at least two different systems: one involving molecules that are transported within the plant and another that uses electrical and/or hydraulic signals to carry the information throughout the entire plant. Both of these information-transmitting systems involve the primary material-transporting systems (xylem and phloem). Furthermore, the signals themselves vary in identity, diversity, specificity, versatility, rapidity, ubiquity, and locality.

Chemical signals (Tables 1, 2, and 3) and electrical signals are diverse. Chemicals that have been implicated in short- and long-distance and/or organism-to-organism signaling include ions (Ca^{2+}, H^+), volatiles (ethylene, methyl jasmonate), and both small (IAA, GA, NO) and large molecules (proteins, RNA). Electrical signals are less diverse and only three (action potentials, variation potentials, and system potentials) have been described. These signals are more or less specific to the type of stimulation. In many instances, signal production and/or transmission depends upon the strength (injurious or non-injurious) and/or type of the stress (biotic or abiotic). As pointed by Davies (2004), plant signal versatility could result from the initial inability of plants to identify the agent (biotic or abiotic) that

Table 2 Identity/diversity of plant molecular signals

Signal	References
Inorganic molecules/ions	
CO_2	Lake et al. (2002) and Kim et al. (2010)
NO	Molassiotis et al. (2010) and Beaudoin (2011)
ROS, H_2O_2	Mittler et al. (2011) and Suzuki et al. (2012)
Ca^{2+}	Batistič and Kudla (2012) and Kurusu et al. (2013)
H+	Kader and Lindberg (2010)
Others	Liu et al. (2009) and Dinant and Suárez-López (2012)
Small organic molecules	
ABA	Raghavendra et al. (2010) and Nakashima and Yamaguchi-Shinozaki (2013)
IAA	Zhao (2010) and Depuydt and Hardtke (2011)
CK	Choi et al. (2011), Ha et al. (2012), and Hwang et al. (2012)
GA	Lau and Deng (2010) and Hauvermale et al. (2012)
Ethylene	Zhao and Guo (2011) and Wang et al. (2013)
Methyl jasmonate	Staswick (2008) and Wasternack and Hause (2013)
Phosphoinositides, diacylglycerol, lipids	Boss and Im (2012), Dong et al. (2012), Guelette et al. (2012), and Benning et al. (2012)
Phenolic compounds	Mandal et al. (2010)
ATP	Tanaka et al. (2010), Chivasa and Slabas (2012), and Sun et al. (2012)
Salicylic acid, methyl salicylate	Hayata et al. (2010) and Shah and Zeier (2013)
Sugars	Wingler and Roitsch (2008) and Eveland and Jackson (2012)
Volatile organic compounds	Heil and Ton (2008)
Large organic molecules	
Oligosaccharides	Shibuya and Nimami (2001)
RNA/miRNA	Kehr and Buhtz (2008) and Sunkar et al. (2012)
Polypeptides	Matsubayashi and Sakagami (2006), Wang and Fiers (2010), and Sun et al. (2011)

Table 3 Diversity of phloem exudate transcripts and proteins from white lupin

Protein identity	Phloem exudate transcript (%)	Phloem exudate proteins (%)
Metabolism	15.3	23.8
Photosynthesis	3	3.8
Protein modification/turnover	10.5	9.2
Redox regulation	4.7	8.5
Signaling	5.4	2
Stress and defense response	5.5	6.2
Nucleic acid binding/transport	7	2.3
Cell structural components	2.6	6.2
Viral	3	ND
Unknown and unclassified	43	37

Table generated using data from Rodriguez-Medina et al. (2011)

causes the insult. As a consequence, a signal may spread throughout the plant to elicit a general stress response. The ambivalence between specificity and versatility is not yet fully understood and it is quite conceivable that this question has only minor significance from a plant's perspective.

While some signals should be transmitted throughout the plant (i.e., for a global response or to coordinate the different growth sites), other signals are restricted to the cells adjacent to the stimulation site when, for example, they evoke necrosis of tissue. Signals, therefore, differ in the distance they are transmitted, thereby resulting in local vs. systemic responses.

For cell-to-cell transport, chemicals must travel through the plasmodesmata and symplast, while for inter-organ transport they go primarily through the vascular system (phloem or xylem). The rate of transport is not much a problem when the signaling occurs over short distances, but it becomes crucial in the case of long-distance transmission. Indeed, signals move at different speeds, mainly as a function of their nature (i.e., chemical or physical). The transport of solute molecules is often relatively slow, limited to the speed of diffusion and to phloem sieve element (SE) fluxes, although faster transport has been reported in association with a hydraulic surge in the xylem (Malone et al. 1994; Hlaváčková and

Nauš 2007). Indeed, the phloem seems to carry a wide variety of signaling molecules (Dinant and Suárez-López 2012). In this case it is worth noting that the persistence of the signaling molecule might last for a relatively long period of time (up to several days). Thus, molecular signaling is often a slow, diffusion-based, long-term, sustainable response, whereas electrical and hydraulic signaling is rapid but transient.

Another kind of signal termed "memory" exists in plants, which often show a temporal delay in response to specific stimuli. Such a system was first observed with the pioneering work of Thellier et al. (1982) and Desbiez et al. (1984). They studied the long-term (several days) storage of a signal evoked by a minor wound that was able to modify plant morphology (bud outgrowth) when the plant was transferred to the appropriate culture medium. It was also observed with different plants that a primary infection leading to systemic acquired resistance (SAR) in the plant provides a long-term (lifelong) memory of the infection (Spoel and Dong 2012) that enhances the plant's resistance to further infections.

In addition to the signaling mechanisms that stay within the same plant generation, a totally different system, known as epigenetic memory (Saze 2008), transfers the increased resistance potential to the next generation, improving the offspring's capacity to survive various kinds of stress (Mirouze and Paszkowski 2011). This intergenerational memory transmission (Molinier et al. 2006) includes enhanced resistance to infection (Luna et al. 2012; Rasmann et al. 2012; Slaughter et al. 2012) and is based on epigenetic mechanisms (Chinnusamy and Zhu 2009; Pastor et al. 2012) that include chromatin remodeling, DNA methylation, histone methylation and acetylation (Chen and Tiana 2007), and small (mobile) silencing RNA (Molnar et al. 2010). Thellier and Lüttge (2013) reviewed the memory processes in plants and proposed an integrative model.

Signal Identity

Plant signaling systems employ multiple pathways and transmission distances (Table 1). Very-short-distance signaling occurs within the cell (e.g., from the cell membrane to the nucleus), involving several different mediators such as calcium, pH changes, membrane derivatives, and phosphorylation. These events elicit metabolic changes that could be transmitted to the adjacent cells through the symplasm and/or apoplasm (Bloemendal and Kück 2013). Short-distance, cell-to-cell transport is especially important in cell regulation including the maintenance of cell identity in the shoot apical meristem (Clark 2001). It involves mainly small molecules (e.g., ions, inositol phosphate – IP3, auxin, ABA), sugars, and oligosaccharides (Shibuya and Nimami 2001), while peptides are crucial in other aspects of plant development (Murphy et al. 2012). It is still unclear if symplastic pathways could account for long-distance molecule transport. Recently, Sokołowska and Zagórska-Marek (2012) showed that symplastic transport of fluorescent tracers could occur between the cambium and living cells in the secondary xylem in *Acer* and *Populus*. The apoplasm seems to allow transmission of signals, especially the ROS wave (Miller et al. 2009; Mittler et al. 2011). It is also the site of short-distance eATP (external ATP) signaling through the activity of apyrase. As an example, cotton fiber growth is tightly regulated through ectoapyrase activity (Clark et al. 2010). The apoplasm contains numerous protein species that can lead to the generation of local signals, including proteases, cell wall–modifying enzymes, and oxidoreductases (Charmont et al. 2005).

In the present chapter, we will concentrate on long-range, inter-organ signal transmission. The research in this area has been marked with considerable efforts to identify the physical basis of the information. It appears that at least two very different yet complementary systems exist: (1) a chemical system based on the movement of molecules and (2) physical systems based on electrical and hydraulic waves.

These signals move mainly using the conducting system of plants (xylem and phloem). The

phloem flow velocities are relatively high, ranging from 1.15 m · h⁻¹ in *Phaseolus* and *Cucurbita* plants (Mullendorea et al. 2010) to 3 m · h⁻¹ in *Ricinus* (Köckenberger et al. 1997). These speeds are consistent with the systemic transport of solutes throughout most herbaceous plants in less than 30 min. In contrast, Chu et al. (2009) showed in *Pachira macrocarpa* that the xylem flow rate increased under wind stress but did not exceed 36 mm · h⁻¹, 50- to 80-fold slower than in the phloem. Indeed, many stress-related signal molecules (salicylic acid, methyl jasmonate) are found in the xylem (Atkins and Smith 2007; Krishnan et al. 2011) either after initial loading or after translocation from the phloem (Rocher et al. 2006).

Molecular-Based Systems

This part of the signaling system is conceptually the simplest one: the information is contained within a chemical that is produced locally in response to the perception of a stimulus and spread to other parts of the plant, as the source of information and, as a consequence, permitting the systemic responses in distant, unstimulated tissues. According to Heil and Ton (2008), these signal molecules must fulfill four criteria. They must be (1) generated locally at the site of stimulation, (2) transported systemically, (3) accumulated in the distant tissue, and (4) capable of evoking a distant response.

Table 2 summarizes some of the molecules that act as long-distance signals in plants and is based on the chemical structure: inorganic molecules and ions, small organic molecules, and large and complex organic molecules.

Local Ionic and Inorganic Signals

Although the bulk of this chapter deals with systemic signals, we will give brief mention to those that have limited transport – mainly from outside the cytoplasm to inside and vice versa. Calcium is a well-known intracellular messenger (Kudla et al. 2010; Batistič and Kudla 2012), which has a very low (10^{-7} M) steady-state level in the cyto-

plasm and orders of magnitude higher in the cell wall. Cytoplasmic calcium is capable of undergoing a rapid and transient increase in concentration in response to a wide variety of stimuli. This increase modulates the activity of several calcium-binding proteins (calmodulin, protein kinases) that induce subsequent cellular events (phosphorylation cascades, modification of enzymatic activities, gene expression). The main challenge remaining is to understand how a single molecule (ion) could mediate such different pathways. It seems that spatiotemporal variations in calcium concentration and interactions with a wide variety of calcium-binding proteins (CDPK, CBL/CIPK, CMLs) constitute the basis to encode these diverse responses (Kudla et al. 2010; Batistič and Kudla 2012).

Protons (H+) are highly important since variations in their concentration affect the cell pH homeostasis and could strongly affect the activity of several enzymes (Kader and Lindberg 2010). Acidification of cytosolic pH (ordinarily at around 7.5) is observed after various kinds of environmental perturbations including wounding (Bonnin et al. 1989) and elicitation of *Eschscholzia* protoplasts with a yeast glycoprotein (Roos et al. 2006). Furthermore, auxin-induced increases in proton concentration in the cell wall can activate several enzymes especially wall-weakening enzymes to stimulate cell enlargement (Schopfer 2006).

Carbon dioxide (CO_2) was recognized as an environmental stimulus able to affect stomatal density, and a signal indicating CO_2 deficiency is generated in the mature leaves and, in conjunction with fatty acids, jasmonate, and oxidizing metabolism is capable of moving to young leaves to control stomatal density (Lake et al. 2002). Carbon dioxide also regulates stomatal aperture through ABA metabolism and a CO_2-binding protein (Kim et al. 2010).

Hydrogen peroxide (H_2O_2) concentration is elevated after many environmental stimuli as a result of movement from the cell wall (Cheng and Song 2006) and is implicated in several cellular functions (phosphorylation, gene expression).

Transmitted Ions and Inorganic Molecules

Nitric oxide (NO), a well-known neurotransmitter in mammals, is also produced in plants after mechanical wounding or insect attack and is implicated in many aspects of plant development (Arasimowicz and Floryszak-Wieczorek 2007; Molassiotis et al. 2010; Beaudoin 2011). It is produced in peas by peroxisomes and is primarily located in the vascular bundle (xylem and phloem), which can serve to transport the molecule throughout the plant (Corpas et al. 2004).

Miller et al. (2009) described the genesis of ROS (reactive oxygen species) in response to various stimuli including wounding, heat, high light, and salt stress. This signal moves rapidly ($8 \text{ cm} \cdot \text{min}^{-1}$) in the apoplasm and can be stopped by the suppression of ROS accumulation. Recently, Mittler et al. (2011) showed the propagation of an ROS wave in response to wounding that spreads from the base to the apex of an *Arabidopsis* plant within 10 min. This auto-propagating ROS signal that moves bidirectionally causes ROS production at a distance from the stimulus site and thus evokes a systematic signaling cascade that follows the production of ROS. It is however not clear if the ROS wave is induced as a generic stress (i.e., nonspecific) signal or if it acts with a specificity that could be encoded through calcium oscillations or protein kinase activity.

Transmitted Simple, Small Organic Molecules

Plant growth regulators (hormones) have long been identified as potential long-distance signals that regulate plant development (Depuydt and Hardtke 2011), although in most cases it remains difficult to establish an unequivocal relationship between the presence of growth regulators in the xylem and/or phloem and morphological and/or metabolic consequences of their translocation (Atkins and Smith 2007).

Abscisic acid or its glucose-ester conjugate (Jiang and Hartung 2008) seems to move from the roots to the stem in water-stressed plants and induces stomatal closure. This molecule appears to be a better candidate for long-distance signal transmission than previously suggested (Peña-Cortés et al. 1991) since a large proportion of ABA could be lost during its transport in the xylem because of the high diffusion of the protonated form, $ABA \cdot H^+$. In contrast, the membrane permeability to the ABA glucose-ester (ABA-GE) conjugate is very low, keeping its concentration constant during transport in the xylem (Jiang and Hartung 2008). The non-active ABA-GE is rapidly cleaved to ABA in the apoplast by a β-glucosidase (Dietz et al. 2000). The ABA signal could be reinforced by sulfate ions that have been found to be more abundant in the xylem sap after water stress in maize (Goodger and Schachtman 2010). Recent advances in the understanding of ABA interaction with phosphatases to enable SNF1-type kinase action (Raghavendra et al. 2010; Nakashima and Yamaguchi-Shinozaki 2013) open new perspectives in the understanding of hormone action.

The long-distance polar transport of auxin (Cho et al. 2007; Zhao 2010) and its transport in the xylem (Wilkinson and Davies 2002) play key roles in plant morphology under stress conditions. Auxin is also identified as a short-distance mobile messenger in root gravitropism (Swarup et al. 2005) and in the maintenance of root stem cells (Ding and Friml 2010).

Cytokinins (CKs) are implicated in plant responses to the environment (Ha et al. 2012). Cytokinin signaling appears complex since CK produced by bacteria has different effects than the CK originating from plants (Choi et al. 2011). Long-distance transport of CK is implicated in the regulation of stomatal aperture (Jia and Zhang 2008) and in nitrogen starvation signaling (Tamaki and Mercier 2007).

Gibberellins are also present in the phloem (Dinant and Suárez-López 2012) and widely implicated in vegetative plant development (Hauvermale et al. 2012). They are likely to remain intact during transport and modulate plant development.

Flooding increases the synthesis of the ethylene precursor, ACC, in the root and in the xylem sap (Malladi and Burns 2007), causing leaf epinasty. Thus, ACC acts as a soluble mobile signal from roots to shoot (Jackson 2002). This internal

long-distance ACC signal could take place in parallel to plant-to-plant airborne transmission of ethylene which is a highly diffusible, volatile compound that elicits and regulates many metabolic processes (fruit ripening, senescence, abscission) and plant responses to stress (reviewed in Bleecker and Kende 2000).

An animal nerve compound, gamma-aminobutyric acid (GABA), a nonprotein amino acid, was proposed as a possible long-distance signaling molecule that upregulates nitrate uptake under conditions of nitrate deprivation (Beuve et al. 2004). However, measuring GABA in the phloem is difficult and the reality of long-distance transport was recently questioned (Shelp 2012).

Systemic responses observed after mechanical wounding rely mainly on the rapid and massive synthesis of jasmonic acid (Hlaváčková and Nauš 2007) and its translocation in the phloem. The synthesis of methyl jasmonate is also observed and it is transported in both the xylem and phloem (Heil and Ton 2008).

Flavonoids are very common, highly diversified phenolic secondary metabolites that are implicated in many metabolic pathways in plants, including resistance to pathogen attacks (Lattanzio et al. 2006). These compounds are transported long distances by using either the symplastic pathway in the upward direction or vascular bundles in the downward direction (Buer et al. 2007). These movements of flavonoids may have considerable significance through their modulation of growth, branching, and auxin transport (reviewed in Buer et al. 2010), although additional work is required to understand these mechanisms.

When plants are attacked by pathogens, they activate a resistance mechanism (systemic acquired resistance, SAR) that operates both locally (at the site of infection) and systemically (throughout the whole plant). Salicylic acid was identified as an inducer of SAR responses both locally and in distant tissue and was therefore proposed as being the mobile chemical transported through the plant to evoke SAR at a distance (Molders et al. 1996). However, it was noticed that SAR could be turned on before sali-

cylic acid increased in the distant tissues and that the genuine signal molecule was in fact methyl salicylate (Park et al. 2007). It was recently demonstrated that light strongly interferes with this signal molecule (Liu et al. 2011) which became unnecessary under high-light conditions (Attaran et al. 2009).

ATP, the main energy molecule of cells, is also a short-distance, signaling molecule when it is exported as eATP (external ATP) outside of the cell (Demidchik et al. 2003) through a leak occurring after cell wall damage (Jeter et al. 2004) or after exocytosis through vesicle fusion (Kim et al. 2006). External ATP, whose presence in the extracellular matrix is tightly controlled through apyrase activity (Sun et al. 2012), could trigger an increase in cytoplasmic $[Ca^{2+}]$ that evokes the accumulation of several transcripts such as MAPK and also in ethylene biosynthesis (Jeter et al. 2004). External ATP also causes the opening of stomata (Hao et al. 2012) and induces the production of nitric oxide in suspension cells (Foresi et al. 2007) that could move in the plant through the vascular bundles. Riewe et al. (2008) demonstrated that apyrase activity in potato is crucial in several aspects of plant development (growth and tuber formation). However, no clear evidence has been given demonstrating long-distance eATP transport in plants.

Sugars are now seen not only as nutrients but also as signaling molecules (Rolland et al. 2006). Sugars are transported mainly as sucrose, whose cleavage takes place in the cytoplasm through the activity of invertase. Hexokinase is the main glucose sensor of the cell, integrating not only sugar but also hormonal signals (Tuteja and Sopory 2008). Indeed, Hammond and White (2011) pointed out a role for sugar and phytohormones (auxin and cytokinin) in changes in long-distance root morphology and phosphate transporter synthesis in root responses to low phosphorus availability. Furthermore, a recent review (Eveland and Jackson 2012) pointed out the potential role for sugars in short-distance signaling (as a key factor in the maintenance of SAM cell identity) and long-distance signaling (in coordination with the plant hormones ethylene, abscisic acid, and

auxin) in the regulation of transcription, translation, and protein activity that control plant growth.

This plethora of different systems within the plant is complemented by plant-to-plant communication, i.e., the ability to send a message from one plant to another through the airborne transmission of volatile organic compounds (VOCs) such as methyl jasmonate, methyl salicylate, and several terpenes (Yi et al. 2009; Arimura et al. 2011). Airborne signaling seems to physiologically precede vascular signaling (Heil and Ton 2008). The airborne diffusion of VOCs eliminates the need for an *in planta* transport system (Frost et al. 2007) and could affect plants at a considerable distance (Frost et al. 2008). Several volatile molecules could act as stimuli to elicit responses in distant plants (Heil and Karban 2009; Ueda et al. 2012). For instance, ethylene, methyl salicylate, methyl jasmonate, cis-jasmone, and several terpenoids alone or in combination are able to enhance resistance of plants to herbivore attacks. Interestingly, the methanol vapor surge produced in response to wounding in "emitter" plants enhances the resistance to bacterial infections in distant "receiver" plants yet increases tobacco mosaic virus reproduction and spreading by increasing cell-to-cell communication (Dorokhov et al. 2012).

Transmitted Complex, Large Organic Molecules

In addition to small molecules, larger organic molecules (oligosaccharides, lipids, peptides, nucleic acids, and proteins) are also transmitted, mainly through the phloem, in response to various stimuli (Wu et al. 2002) and contribute to systemic signaling (Fukuda and Higashiyama 2011).

Oligosaccharides were recognized a long time ago as potential elicitors for stress responses (reviewed by Shibuya and Nimami 2001). They could originate from the cell wall of pathogens (β-glucan oligosaccharides, chitin- and chitosan-derived oligosaccharides) or from the damaged cell wall of plants (oligogalacturonides) and are mainly active in dicots. Large oligosaccharides (up to 20 residues) are the most active ones, evok-

ing the accumulation of phytoalexins, defense proteins such as protease inhibitors, the lignification of the attacked regions (but not the hypersensitive response-induced tissue necrosis), and promotion of the antioxidant system in alfalfa roots (Camejo et al. 2012). It is not clear if these compounds contribute to long-distance signaling. Baydoun and Fry (1985), using radiolabeled oligosaccharides, demonstrated that only the small (up to 6 residues), moderately active oligosaccharides were mobile while the larger, more biologically active ones stay close to the site of stimulation. However, Iwai et al. (2003) demonstrated that the xylem sap actually contains oligosaccharides, oligoglycans, and oligogalacturonides that may be involved in long-distance signaling. Simpler molecules such as glycans and sugars also appear to have a signaling function (Lalonde et al. 1999; Etzler and Esko 2009) because of their rapid transport in the phloem.

Recent reports have pointed out the presence of lipids in the phloem (Benning et al. 2012), including phytosterols, glycolipids, and fatty acids, which are not the result of membrane degradation. Some of them are known to be involved in transduction pathways after different environmental stimuli such as wounding, pathogen attacks, and flaming (Guelette et al. 2012). These include phosphatidic acid, phosphatidylglycerol, and phosphatidylinositol that could originate from membranes after lipase activity. They could, in addition to their signal transduction function, themselves be important long-distance signaling molecules to evoke distant responses if their activity is kept intact after being transported. Others, such as jasmonic acid or oxylipin, are specifically produced in response to pathogen infection and are transported in the phloem (Schilmiller and Howe 2005). Lipids are associated with specific proteins (phloem lipid-associated family protein, PLAFP; annexin) that allow the efficient transport of these hydrophobic molecules in the phloem (Benning et al. 2012).

Peptides such as systemin may act as long- or short-distance signaling molecules (Butenko et al. 2009). Systemin, an 18 amino acid peptide, derived from a 200 amino acid precursor prosys-

temin, isolated from wounded tomato leaves, is able to trigger the systemic expression of protease inhibitor (PI) genes that protect the plant from insect attack (Pearce et al. 1991). Synthetic systemin could be transported in the phloem and was proposed to be the mobile signal responsible for the systemic response (McGurl et al. 1992; Narváez-Vásquez et al. 1994). Jasmonic acid (JA) evokes the expression of protease inhibitor genes (Farmer and Ryan 1990) and is required to initiate the systemic response since the JA-deficient *spr-1* mutant is impaired in the genesis of the systemic signal while maintaining the local response to wounding. Jasmonic acid (whose synthesis is induced by systemin) appears to be the transmitted wound signal (Lee and Howe 2003; Sun et al. 2011). A similarly small (23 amino acid) peptide that triggers systemic wound responses was also identified in *Arabidopsis* as AtPep1 (Huffaker et al. 2006). This peptide binds to membrane receptors (Yamaguchi et al. 2006, 2010) identified as LRR receptor kinases involved in plant responses to aggression. Similar receptors are involved in the binding of phytosulfokine (Matsubayashi et al. 2006), another plant peptide that promotes cellular differentiation and could act as a signaling molecule to reduce (or induce) stress responses (Motose et al. 2009). RALF (rapid alkalinization factor), a 5-kDa peptide (Pearce et al. 2001) that inhibits root growth, may be transported in the xylem sap (Neumann 2007) and constitute a putative long-distance signal molecule. In contrast, the well-characterized CLAVATA3/ESR (CLE) peptide-based signaling pathway only operates for short distances to maintain cell identity in shoot apical meristems (Juna et al. 2008).

RNA can move from cell to cell through the plasmodesmata (Wu et al. 2002) and diffuse short distances. A wide variety of mRNAs (implicated in housekeeping, pathogen resistance, and stress responses) were also found in the phloem (Kehr and Buhtz 2008; Lee and Cui 2009). The phloem SE do not contain translation machinery, and so the different nucleic acids types (mRNA and small RNA) are being transported to distant tissues where they can be translated or influence metabolic activity (Kim et al. 2001; Kehr and

Buhtz 2008). Untranslated transcripts are likely to bind to transport proteins (which are consistently detected in the phloem sap). A model for RNA trafficking was recently proposed by Lee and Cui (2009), who suggested that chaperons and mRNA-binding proteins are implicated in mRNA loading into the phloem. The translocation of these mRNAs inducing developmental changes was reported more than 10 years ago (Ruiz-Medrano et al. 1999; Kim et al. 2001), and they have an active role in leaf morphogenesis (Haywood et al. 2005) and tuber formation (Banerjee et al. 2006).

The diversity of phloem mRNA was assayed in *Ricinus* (Doering-Saad et al. 2006) and revealed over 150 unique transcripts. In potato, Hannapel (2010) demonstrated that a StBEL5 mRNA is transported a long distance to the stolon tip where it regulates tuber formation. It seems important to assess the differential phloem transcriptome between control and stimulated plants (both in local and distant tissues) to assess the diversity of mRNAs transported in the phloem that specifically act as signals. Classical molecular methods (i.e., subtractive cloning or global sequencing) could provide valuable information to explore the importance of mRNA trafficking in systemic plant responses.

The phloem sap also contains noncoding small RNAs (Yoo et al. 2004; Buhtz et al. 2008) that could interfere with translation (Zhang et al. 2009). Micro RNAs (miRNA) are small, non-translated RNAs that regulate gene expression post-transcriptionally (Yoo et al. 2004). They accumulate after various environmental stimuli such as nutrient deficiency (Fujii et al. 2005), wounding (Bozorov et al. 2012), or pathogen attack (Feng et al. 2011). These RNAs are present in the phloem sap and are readily mobile (Buhtz et al. 2010). It is widely admitted that miRNAs play a major role in long-distance information transfer. MicroRNA399 appears to be involved in phosphate starvation (Pant et al. 2008), while Buhtz et al. (2008) identified three miRNAs that accumulate in the phloem when plants were cultivated in conditions deprived of ions. In this context, a challenging task is to identify the targets of these miRNAs since the classical approach by

sequence alignment is not sufficiently accurate and leaves a high proportion of nonsignificant putative interactions, mainly because these computerized analyses are performed with little reference to the ongoing metabolism.

The phloem also contains a diverse array of proteins (Kehr 2006) including calcium-related proteins (annexins, calmodulin, kinases), ROS actors (ascorbate peroxidase, glutathione-S-transferase, thioredoxins), protease inhibitors, and lectins, all of which are potential long-distance signal molecules. It is not clear however if the proteins are themselves signals or if they contribute to signal propagation through their metabolic activity. Kehr (2006) compiled data showing that phloem sap contains a large variety of stress-related proteins, including redox metabolism and calcium-related proteins. Giavalisco et al. (2006) characterized the phloem sap of *Brassica napus* and found over 140 protein species, some of them related to redox/stress metabolism and signal transduction, and a large set of metabolic enzymes (e.g., UDP glucose pyrophosphorylase, glyceraldehyde-3-phosphate dehydrogenase, malate dehydrogenase). Recently, Rodriguez-Medina et al. (2011) described the proteome and transcriptome of white lupin phloem sap and found numerous molecules that are similar to those previously described. However, more than one-third of them remain unknown, but the others were categorized into families: basic metabolism, structural components, nucleic acid binding, and stress-related response including redox regulation (Table 3), indicating a wide variety of potential metabolic activities. This latter point is of major interest since the phloem is now understood as a transport system that possesses its own set of proteins and transcripts that are likely to play a central role in systemic responses of plants in response to environmental stresses.

This diversity of protein and enzymatic activity was recently confirmed in *Arabidopsis* by Batailler et al. (2012) where 127 proteins were identified as belonging to pathways involving carbohydrate, lipid, hormone, amino acid, nucleotide, and secondary metabolism, as well as gluconeogenesis, oxidative stress, and the tricarboxylic acid cycle. Aside from these enzymes, the phloem sap also contains all the species (protein transport, cytoskeleton, defense proteins, etc.) that were identified in previous work. The authors strongly suggest that very active metabolic activity takes place in the phloem SE.

Physical Systems

It was pointed out over 30 years ago (Davies and Schuster 1981) that such molecular-based systems fail to explain the rapid, long-distance transmission of signals whose speed is many orders of magnitude greater than chemical transport processes, and so efforts were made to identify the nature of such rapidly generated, bidirectionally transmitted signals (Davies 1987). In the last two decades, it has been shown that, in addition to the chemical signals described above, plants also possess two physically based signals, electrical and hydraulic, and these differ substantially from the chemical-based systems (reviewed in Davies 2006). They are fully systemic, traveling from the root to the stem (and stem to root), exceedingly rapid, and most likely possessing a limited information capacity. Indeed, it seems most likely that the more rapidly a signal is generated and transmitted, the less its information content (Davies and Stankovic 2006).

Electrical Signals

Although electrical signals have been known for over 200 years (Pickard 1973; Davies 2006 and references therein), it is only recently that valid functions have been assigned to them. Indeed, 25 years ago it was still necessary to hypothesize a function (Davies 1987), although rapidly generated, bidirectionally transmitted signals were already known to evoke changes in translation (Davies and Schuster 1981). Electrical signals are now known to affect many aspects of metabolism, including RNA synthesis (Stankovic and Davies 1996, 1997), protein synthesis (Davies and Stankovic 2006), and photosynthesis (Grams et al. 2009).

There are at least four different, yet sometimes intertwined, electrical signals, action potentials (AP), variation potentials (VP), system potentials (SP), and voltage transients (VT), and here we will focus on the first two (reviewed in Davies 2006; Fromm and Lautner 2007). Action potentials are genuine electrical signals, i.e., all-or-none, self-propagating (via the flux of ions through voltage-gated channels), almost constant in velocity and magnitude, and have a refractory period during which a subsequent AP cannot be generated (Davies 2006). In contrast, variation potentials are not genuine electrical signals but local changes in membrane potential evoked by changes in the hydraulic status of the tissue acting on pressure-sensitive ion channels and vary in apparent magnitude and velocity (Davies 2006). Nevertheless, they both seem to involve the same ions, being initiated by an influx of Ca^{2+} and then an efflux of Cl^- and K^+, but they differ in the magnitude and duration of these fluxes (Davies 2006) and in some instances proton fluxes are also involved in long-distance electrical signaling (Grams et al. 2009). Although any cell with voltage-gated channels can generate and transmit signals from cell to cell (via plasmodesmata), long-distance transmission seems to be exclusive through the phloem, primarily the phloem SE (Van Bel et al. 2011; Hafke and Van Bel 2013); thus, methods for "tapping" the phloem are important for electrical as well as chemical signals. APs can be evoked by any external stimulus (electrical, cold, heat, wound) or internal stimulus that can elicit a change in membrane potential sufficiently large to activate voltage-gated channels (Davies 2006).

The rates of transmission of the electrical signals are also variable. The action potential in *Mimosa pudica* propagates rapidly, at a rate of $20–30 \text{ mm} \cdot s^{-1}$ (Fromm and Lautner 2007). In sunflower plants, the action potential propagates at a rate of about $7–10 \text{ cm} \cdot min^{-1}$, whereas the variation potential has an initial velocity of almost $30 \text{ cm} \cdot min^{-1}$, rapidly decreasing further from the wounded region to 10–20 % of its initial value (Stankovic et al. 1998).

Hydraulic Signals

According to Malone (1993) hydraulic signals are ubiquitous in plants and can pass through the hydraulic continuum (mainly the xylem) exceedingly rapidly, especially after wounding (disruption of the integrity of the xylem). Indeed, the rate of water movement in the xylem under these conditions can exceed 10 mm/s, while the movement of the pressure front (loss of tension in the xylem) can equal the speed of sound (1,500 m/s). The xylem, therefore, furnishes a multipurpose signaling system. First, the chemicals (e.g., ABA, PIIF) synthesized in or released from the surrounding cut tissue can act as systemic signals with velocities approaching 10 mm/s. Second, changes in pressure will be sensed throughout the xylem and transferred to adjacent living cells as a change in hydrostatic pressure, which, in turn, can affect pressure-sensitive channels in the phloem. Third, the changes in ion flux as a result of these channel openings can be converted into an electrical signal. Fourth, this signal will appear to be a self-propagating signal but is actually the result of local changes in membrane potential accompanying the self-propagating loss of tension. Thus, the loss of tension will diminish with distance from the wounding site and the accompanying electrical changes will diminish, and the resulting signal varies and is thus referred to as a variation potential (VP). In some circumstances the local changes in membrane potential can trigger an action potential (AP), the self-propagating electrical signal. The interplay between the xylem and phloem as long-distance conduits of information and local signaling through the adjacent parenchyma and companion cells is shown very nicely in Fig. 1 (from Fromm and Lautner 2007). It is not surprising that VPs can evoke at least as many downstream consequences as can APs, since both involve influx of Ca^{2+} and efflux of Cl^-, K^+, and perhaps H^+, but these changes in ion concentrations are greater and longer lasting with VPs than with APs. As stated earlier these altered levels of ions in both the symplast and the apoplast can evoke a plethora of changes (Davies 1987; Fromm and Lautner 2007). See Table 4
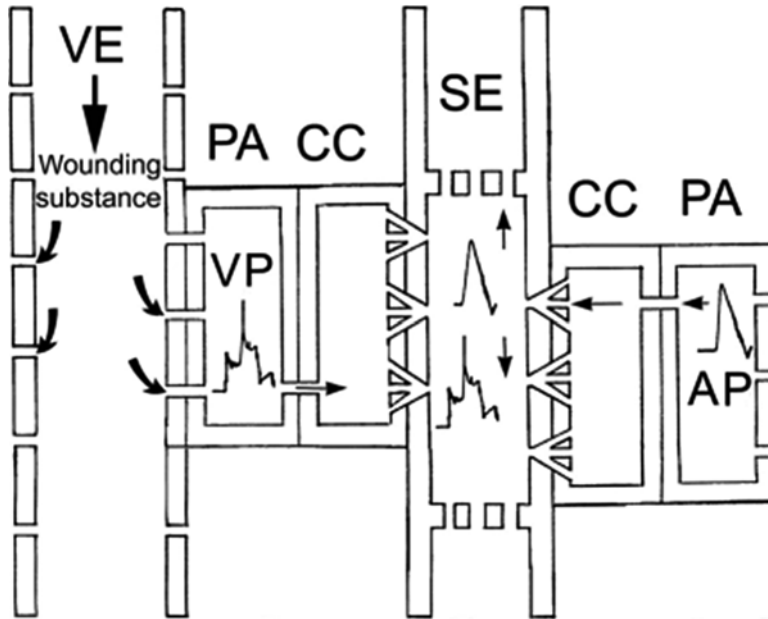
Fig. 1 Electrical communication over long distances. An AP (*right*) can propagate over short distances through plasmodesmata, and after it has reached the sieve element/ companion cell (SE/CC) complex, it can travel over long distances along the SE plasma membrane in both directions. In contrast, a VP is generated at the plasma membrane of parenchyma cells (PAs) adjacent to xylem vessels (VEs) by a hydraulic wave or a wounding substance. Because VPs were measured in SEs (Lautner et al. 2005), it is suggested that they also can pass through the plasmodesmal network and can reach the phloem pathway. However, in contrast to APs, their amplitude will be reduced with increasing distance from the site of generation (Figure 3 from Fromm and Lautner (2007). Copyright John Wiley and Sons. Reproduced with permission)

(from Fromm and Lautner 2007 for a list of well-documented responses to electrical signals). Interestingly, it appears that the hydraulic and electrical components can work independently of each of other during the relatively non-damaging treatment of re-irrigation of maize (Grams et al. 2007).

Methods of Analysis and Possible Applications in Agriculture

Molecular-Based Signals

We will concentrate on metabolites present in the phloem since it is the major route for long-distance transport of signaling molecules (Dinant and Lemoine 2010) and thus the most promising and accessible point of investigation to increase overall knowledge and develop practical applica-tions to agriculture. The challenges to fulfill are fourfold: (1) efficient and selective collection of the circulating phloem sap, (2) analysis of its contents, (3) understanding and reconstruction of the metabolic pathways that could take place in the phloem, and (4) testing of individual metabo-lites or combinations for their effects on plant tolerance to diverse environmental stresses.

Phloem Sampling

First of all, the diversity of molecules that are present before and after the application of a stim-ulus that elicits a systemic response needs to be assayed as completely as possible, both at the site of stimulus perception and in distant tissues. This step relies on an efficient and selective phloem sampling method. This is a difficult task since the phloem SEs are located within the stem and they possess self-repair mechanisms in case of injury. The phloem is highly reactive to damage (Kehr

Table 4 Well-documented physiological effects of electrical signals in plants (From Fromm and Lautner 2007)

Stimulus	Signal	Plant	Physiological effect	Reference(s)
Mechanical	AP	*Dionaea*	Trap closure Release of digestive enzymes	Sibaoka (1969)
Mechanical	AP	*Drosera*	Tentacle movement to wrap around the insect	Williams and Pickard (1972a, b)
Cold shock, mechanical	AP	*Mimosa*	Regulation of leaf movement	Fromm and Eschrich (1988a, b, c) and Sibaoka (1966, 1969)
Electrical	AP	*Chara*	Cessation of cytoplasmic streaming	Hayama et al. (1979)
Electrical	AP	*Conocephalum*	Increase in respiration	Dziubinska et al. (1989)
Pollination	AP	*Incarvillea*, *Hibiscus*	Increase in respiration	Sinyukhin and Britikov (1967) and Fromm et al. (1995)
Re-irrigation	AP	*Zea*	Increase in gas exchange	Fromm and Fei (1998)
Cold shock	AP	*Zea*	Reduction in phloem transport	Fromm and Bauer (1994)
Electrical, cooling	AP	*Luffa*	Decrease of elongation growth of the stem	Shiina and Tazawa (1986)
Electrical	AP	*Lycopersicon*	Induction of *pin2* gene expression	Stankovic and Davies (1996)
Heating	VP			
Heating	VP	*Vicia*	Increase in respiration	Filek and Koscielniak (1997)
Heating	VP	*Solanum*	Induction of jasmonic acid biosynthesis and *pin2* gene expression	Fisahn et al. (2004)
Wounding	VP	*Pisum*	Inhibition of protein synthesis, formation of polysomes	Davies et al. (1986) and Davies and Stankovic (2006)
Heating	VP	*Mimosa, Populus*	Transient reduction of photosynthesis	Koziolek et al. (2004) and Lautner et al. (2005)

et al. 2005) and does so by the activation of anti-oxidant metabolism and the mobilization of P-proteins and callose to repair the damage and plug the intercellular connections. Thus, one needs to keep in mind that the necessary intrusion into the phloem tubes to collect the sap is likely to evoke the accumulation of defense-related molecules. The three methods that were developed to sample the phloem content were recently reviewed from a technical point of view (Dinant and Kehr 2013). All these methods have the same limitation (i.e., they require physical intrusion of the plant tissues that could be perceived as a wound, and therefore modify the steady-state content of undamaged phloem tubes). The spontaneous exudation is the oldest and the simplest one to apply since it consists of the collection of phloem SE contents after localized incision with a razor blade or syringe needle. While this method allows recovery of relatively large amount of phloem SE fluid from plants such as palm and pumpkin, it remains difficult to prepare pure (unpolluted) samples and the rapid accumulation of P-proteins and callose in the conducting tubes prevents the release of SE contents in numerous plants. The use of calcium-chelating agents such as EDTA (EDTA-facilitated exudation) prevents the formation of callose and

P-protein accumulation and thus the closure of phloem tubes (Ernst et al. 2012). In contrast, the use of aphid feeding provides high-quality phloem sampling. The insects that feed on phloem SE contents use their stylet to wend its way between several cells before specifically puncturing the phloem SE and injecting compounds that prevent callose formation, thereby keeping the stylet open. When the stylet is in place, the insect is removed with a laser beam while the stylet is left attached and intact. The phloem SE sap exudate is collected with a glass microcapillary for further analysis. The first drops of sap exudation are usually discarded to prevent pollution (Doering-Saad et al. 2006). While the amount of phloem sap that can be obtained through this method is low, its high quality allows the use of a wide variety of analysis methods and thus should remain the method of choice in herbaceous plants to accurately access and assess the diversity of the phloem content.

Metabolic Activity in the Phloem

Several methods are available to assess the phloem sap content. Fourier transform infrared spectroscopy (FT-IR) is a rapid and versatile method that was successfully used to develop metabolic fingerprints in plants to characterize control vs. salt-stressed tomato (Johnson et al. 2003) or control vs. infected elm (Martína et al. 2005). The method is sufficiently rapid to allow a high number of analyses, and thus the importance of the development stage, diurnal variations, or responses to environmental stimuli can be explored (López-Gresa et al. 2012). These fingerprints could serve as a starting point to develop more sophisticated strategies of metabolome determination. Metabolic profiling aims at the complete description of the different molecular species that are present in a tissue. This technique was successfully used in plants over a decade ago (Fiehn et al. 2000) and is now sensitive enough to assess metabolic diversity on a cellular scale. For instance, micro-sampling of *Arabidopsis* epidermal cells followed by GC-MS-based metabolite profiling allows effective exploration of their metabolome (Ebert et al. 2010). Analytic methods such as high-performance liquid chromatography, gas chromatography, and capillary electrophoresis coupled to mass spectrometry or nuclear magnetic resonance are routinely used to assess metabolome diversity (see Dunn and Ellis 2005; Büscher et al. 2009 for a practical comparison of these methods). Comparative studies of metabolic fingerprinting and/or metabolic profiling of phloem sap from control and stimulated (both local and distant tissues) should provide a very powerful tool to select some metabolites that display differential accumulation. The determination of the phloem protein diversity using classical 1D and 2D gel electrophoresis followed by micro-sequencing or mass spectrometer analysis remains difficult because of the low amount of phloem sap. Strategies using nano-flow liquid chromatography linked to a mass spectrometer (Aki et al. 2008) could constitute a valuable method to assess the phloem proteome.

The phloem sap is an alkaline (pH 8), concentrated solution (osmotic pressure of 1.3–1.5 MPa, Fukumorita and Chino 1982) that may not be optimal for enzyme function. Therefore, the presence of several putative enzymatic activities that are present in the phloem in many unrelated plant species (Kehr et al. 2005) might have two different yet related functions. The first function is the transport of enzymes from their site of synthesis to their site of use via the phloem. This is the simplest explanation since the enzymes and metabolites are considered to be either present or transported "as is" with no interaction between the enzymes and surrounding metabolites. For the second function, the phloem sap is considered as an integrated environment where enzymatic reactions take place and thus modify or transform the molecular signals that originate in the stimulated area. The latter perspective opens new insights for the phloem content to be a metabolic network bearing its own regulation through the general phloem osmotic and ionic environment and the presence of diverse molecules that could interfere with enzymatic reactions. Enzymatic activity in the phloem is demonstrated for oxidative pathways (Walz et al. 2002) and biosynthesis of the plant hormones, ethylene, and jasmonate. It is difficult to decipher which reactions are

likely to take place and how they can modify the dynamic of parallel reactions.

Molecular network modeling could constitute a powerful tool to deal with this complexity. Different approaches exist, mainly employing stoichiometric and kinetic models. Stoichiometric models (Llaneras and Picó 2008) are the easiest to set up insofar as they do not require precise information on the steady-state levels of molecular reactions of the molecular network under consideration. In contrast, kinetic models (Schallau and Junker 2010) use different information (enzyme rate, stoichiometry of the reactions, etc.) to feed a precise dynamic mathematical description of the enzymatic processes. In return, the model predicts the time evolution of the different molecular species concentrations (substrates and products of many parallel reactions that could be partially linked). However, this information is not always available, nor easy to measure, making kinetic models more difficult to set up. Both approaches could be used to get information on metabolic fluxes and network-based pathway analysis. This information could in turn be of great interest to better understand the dynamics and distribution of metabolites in diverse tissues and at various time points.

Different kinds of metabolic models have been developed in plants to model the metabolic network (Kruger and Ratcliffe 2012). As an example, the primary metabolism of *Arabidopsis* was modeled (Gomes de Oliveira Dal'Molin et al. 2010). This model (AraGEM) takes into consideration over 1,500 metabolic reactions in several compartments (cytoplasm, vacuole, peroxisome, etc.) and has proven its utility for the prediction of basic metabolism cycle reactions (e.g., photorespiration) and is robust enough to test in silico functional analysis. Recently, Mintz-Orona et al. (2012) published another model (mainly generated from database information) that takes into account the diverse location of enzymes and metabolite fluxes in *Arabidopsis*. Transferring such methods to phloem metabolism networks should be possible since the number of molecular species and enzymes is likely to be much lower than in the cytoplasm. High-purity phloem sampling followed by metabolome analysis and database contribution should furnish metabolic models useful for predicting molecular events on a spatial (local and distant) and temporal basis, thereby identifying the molecular species that differentiate the control vs. stimulated phloem sap. These molecular species could therefore be used as candidate molecules to test their effect on plant tolerance to environmental stimuli.

Effect of Bioactive Molecules on Plants

The effect of these treatments could then be monitored on some of the traits of interest both for agriculture (e.g., growth, yield, resistance to pathogens, etc.) and horticulture (global architecture, leaf morphology, date of flowering, etc.). The general strategy is presented in Fig. 2. The phloem is sampled and analyzed to determine its proteome (2), metabolome (3), and transcriptome (4) both in tissue adjacent to and distant from the stimulus (1). Proteome and metabolomes account for molecules that are immediately active. In contrast, the transcriptome accounts for translatable (mRNA) or regulatory (miRNA) molecules, although the phloem is devoid of translation equipment. The information from these analyses, combined with databases, feeds a metabolic network model (5) that helps to identify putative molecules of interest. These molecules are therefore used (alone or in combination) to treat plants (6); the effects are observed on different items of interest (e.g., increased resistance to pathogens, crop production in stressed environments, plant architecture) using an automated high-throughput phenotyping facility (7).

This strategy requires a considerable number of plants to perform the analyses because of the diversity in metabolites, combinations, and concentrations (several thousand per test is a reasonable estimate). It is therefore an absolute necessity to use an automated, high-throughput system (phenotyping, Furbank and Tester 2011) to evaluate the effect of treatments on traits of interest. Such automated facilities are now functional and some of them were recently used in a very similar context (Tardieu and Tuberosa
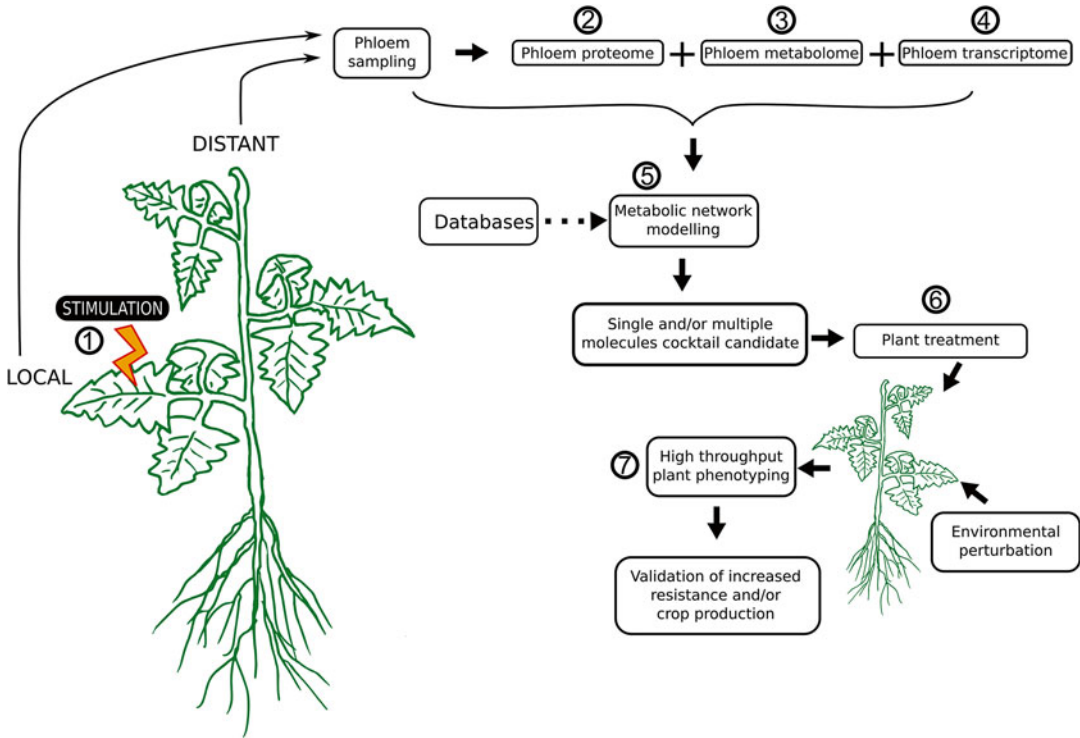
Fig. 2 General strategy to test effect of a molecule (or a combination of molecules) on agricultural or horticultural traits of interest. The plant is stimulated locally to potentially evoke both local and systemic responses (*1*). Phloem sap is sampled and analyzed for proteome (*2*), metabolome (*3*), and transcriptome (*4*). The collected information is used to feed metabolic network modeling (*5*, along with information from databases) to select molecule(s) of interest used to treat plants (*6*) subjected to environmental perturbation. The effect of this treatment (e.g., increased resistance to pathogens, plant architecture, etc.) is evaluated using a high-throughput plant phenotyping facility (*7*)

2010). These authors used a phenotyping facility to identify genetic loci of interest that allow plants to maintain agricultural performances under stress condition (stress tolerance as inheritable traits): the phenotyping facility uses specialized cameras and software (Hartmann et al. 2011) to identify phenotypic variations (e.g., plant architecture) that are related to quantitative trait loci (QTL) of interest. A very similar approach could be used to test the effect of phloem molecules or combinations of molecules (characterized from differential metabolic profiling and/or from metabolic network modeling) on plant characteristics of interest (Fig. 3). These phenotyping methods are applicable to the stem as well as the roots (Iyer-Pascuzzi et al. 2010).

Methods for Electrical-Based Signals

Both of the major electrical signals in plants, action potentials (AP) and variation potentials (VP), must, by definition, involve selective ion movement across membranes leading to changes in membrane potential and thus can be measured by measuring either of these parameters.

Measuring Membrane Potential Using Electrodes

Until recently, the method of (a very limited) choice was to use electrodes, either intracellular or, far more frequently, extracellular, and generally these electrodes measured the difference between the so-called measuring electrode(s) and
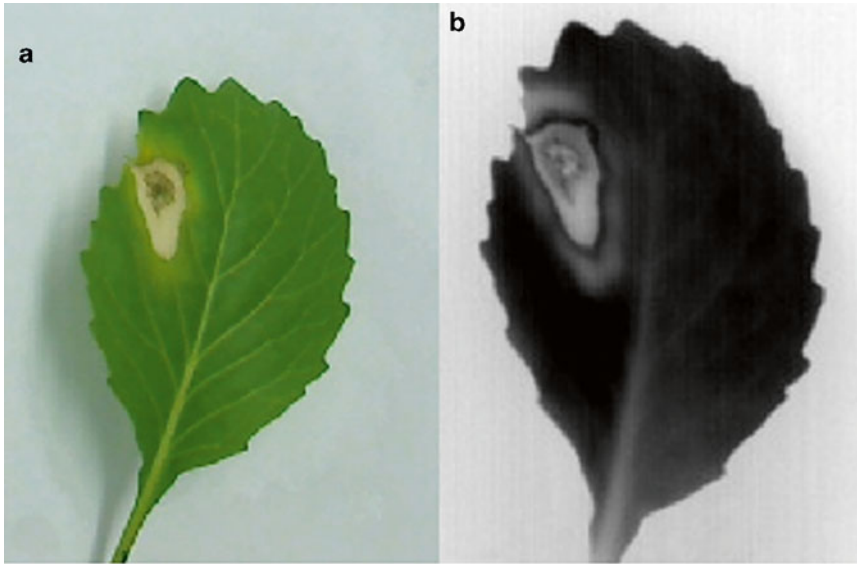
Fig. 3 Example of phenotyping in cabbage after *Alternaria brassicicola* infection. Thermal imaging of a cabbage leaf infected with *Alternaria brassicicola* (5 days after inoculation) to quantify the surface of the leaf infected by the pathogen. (**a**): visible scan image of black spot symptom; (**b**): thermal image. Such images could be use to feed an automated process to identify the potential effect of plant treatment toward an infection (Photographs courtesy of IRHS-FungiSem, obtained with Phenotic technical facilities, SFR 4207 Quasav, Angers, France)

the reference or ground electrode (Davies 2006). The extracellular electrodes measure the global ionic activity within their sphere of influence and can be either surface contact electrodes or inserted electrodes. The former cause little damage to the plant but suffer from drying out and thus a limited duration of use, while the latter damage the tissue but can be used for days or even weeks (Davies 2006). Relatively little skill is needed in conducting these measurements and, except for computer and associated programs for analyzing data, they are comparatively inexpensive. These are the methods we have routinely used, and we eventually chose the extracellular (inserted) electrodes even for experiments on wounding, since we could wait for the plant to recover from the (minor) wound of inserting the electrode before performing the major wound (Stankovic et al. 1998). Since AP and VP have different properties (velocity, magnitude, etc.), it is necessary to have a series of electrodes referenced with the ground electrode in order to differentiate between these signals.

In contrast to extracellular electrodes, intracellular electrodes require far greater skill on the part of the experimenter as well as much more sensitive, delicate, and expensive equipment to make the electrodes, insert them, and conduct the recordings. Work with plants is generally far more difficult than work with animals, and the use of intracellular electrodes is no exception. An animal cell has at most an extracellular matrix surrounding it, which furnishes a minimal physical barrier to an electrode, whereas a plant cell has a tough wall, which can easily break the electrode when attempting to insert it. Furthermore, the animal cell is primarily cytoplasm, whereas the plant cell may be 90 % vacuole and it takes great skill to position the electrode in the cytoplasm and not in the cell wall or the vacuole. Nevertheless, such intracellular electrodes have been use frequently and with great success.

As mentioned earlier, the phloem is a major conduit for chemical and electrical signals but analysis of its contents are made difficult by the wound-induced formation of P-proteins. This has

been very nicely circumvented by the use of aphids, which can make their proboscis wend its way between different cells until it encounters a phloem SE. It then punctures the SE and releases an anti-clogging agent. The aphis is severed, leaving the proboscis in the SE. Not only does this allow the release of chemicals from the phloem but it can also act as a recipient for a microelectrode which can then be used to measure action potentials (or specific ions if necessary). This is shown very nicely in Fig. 4 (from Fromm and Lautner 2007).

Measuring Membrane Potential Using Fluorescence

The use of fluorescence as a means to detect macromolecules and small molecules has been around for a few decades, but the use of highly specific fluorescent proteins gained ascendancy with the work from Roger Tsien's lab (Tsien 1998) on green fluorescent protein (GFP) and the tremendous array of genetically modified versions with different absorption and emission spectra that have been developed since (Tsien 2010). The most recent review of the invaluable
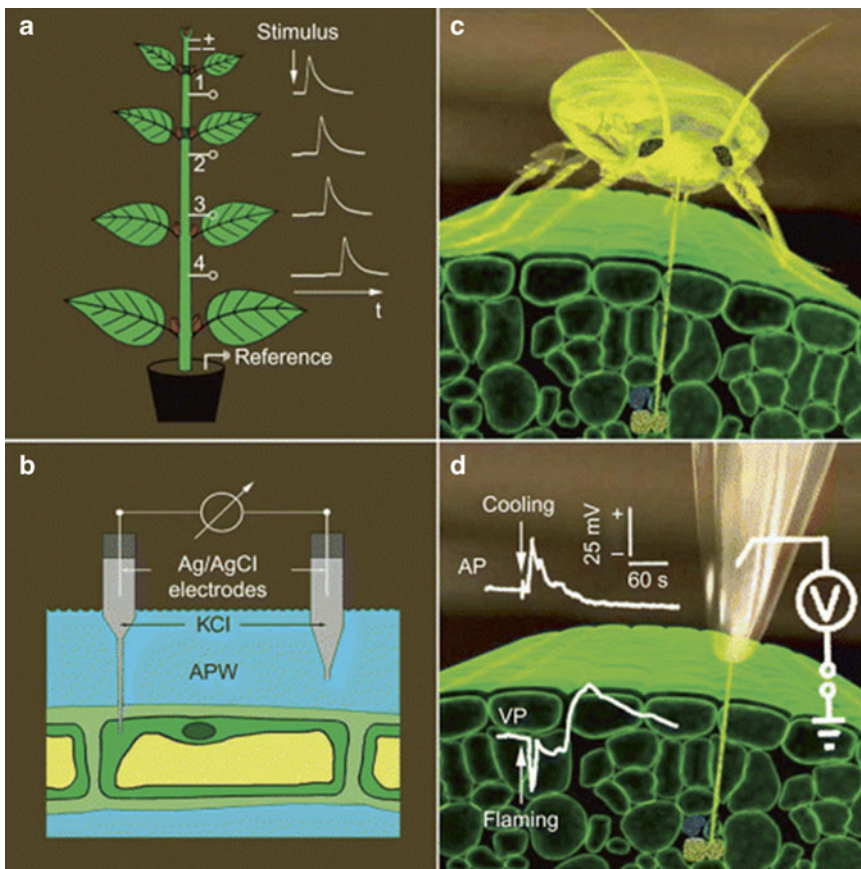
Fig. 4 Techniques for measuring electrical signals in plants. (**a**) Extracellular recording with four channels and a reference electrode inserted in the soil. ±, electrical stimulation. An AP (*right*) generated by electrical stimulation appeared successively at electrodes 1, 2, 3, and 4. (**b**) Intracellular measurement of the membrane potential with a microelectrode inserted into the cytoplasm of an algal cell while the reference electrode is in contact with the artificial pond water (APW) outside the cell. Both electrodes are filled with KCl, clamped in Ag/AgCl pellet holders, and connected to an electrometer. (**c**) Phloem potential measurements; an aphid in feeding position with its stylet inserted into a sieve element on the upper side of a leaf. (**d**) After the aphid is separated from its stylet by a laser pulse, the stylet stump exuded sieve tube sap to which the tip of a microelectrode was attached. Cooling the shoot evoked an AP transmitted acropetally within the phloem, while flaming of a leaf generated a VP with different form and of long duration. *t* time (Figure 1 from Fromm and Lautner (2007). Copyright John Wiley and Sons. Reproduced with permission)

role of fluorescent proteins in plant research (Okumoto et al. 2012) describes their construction, their use in high-resolution imaging *in planta*, and their use in discovery of novel phenomena. More on this topic is included in a companion chapter in this volume (Davies and Stankovic, Plant Cytomics: Novel Methods to View Molecules on the Move).

There has been a recent surge of reports (mainly in *Nature Methods*) describing the use of voltage-sensitive fluorescent proteins to analyze membrane potential and the accompanying action potentials in animal systems. These include the use of fluorescence (or Förster) resonance energy transfer (FRET) of a specially constructed donor and acceptor termed "Mermaid" which was used to record voltage spikes similar to those found with action potentials measured using electrodes (Tsutsui et al. 2008). More recently a bacterial rhodopsin has been shown to "run in reverse" (Looger 2012). This protein is normally activated by light to evoke ion fluxes in vivo, but it can now be used to monitor ion fluxes through voltage-mediated emission of light (Looger 2012). Indeed, these voltage-sensitive fluorescent proteins (VSFP) have now been genetically targeted to specific (animal) cell types (Akemann et al. 2010; Kralj et al. 2011). Pastrana (2012) points out the great value of such techniques in the neurosciences, where immense skill and patience are needed to insert microelectrodes into delicate cells deep within tissues, but far less skill (with perhaps greater accuracy) can be obtained using VSFP. We know of no work with plants using such methods but consider it an excellent path to follow, especially since microelectrode insertion is even trickier with plants. However, it would be difficult to get the appropriate microbial rhodopsin expressed in cells such as phloem SE (lacking translation apparatus) and impossible in xylem vessel elements (dead), which are the major conduits for systemic electrical signals in plants.

Measuring Ion Fluxes Using Electrodes

Both vibrating probes (reviewed in Dorn and Weisenseel 1982) and ion-specific electrodes (reviewed in Ammann 1986 and in Blatt 1991) have been used to determine ion fluxes during action potentials. As with voltage measurements described above, these methods are now being superseded, at least in animal research, by fluorescence techniques.

Measuring Ion Fluxes Using Fluorescence

Fluorescence has been used to measure action potentials (or more specifically the Ca^{2+} influx that takes place during an AP) in plants ever since the development of fluorescent proteins such as the jellyfish protein aequorin (Williamson and Ashley 1982). More recently green fluorescent protein (GFP) and its extended family (Tsien 1998, 2010) have found multiple uses, including in plants (Haseloff 1998), and have come to the fore in calcium measurements, especially in the field of neurophysiology, and again the most relevant articles appear primarily in *Nature Methods*. Genetically modified calcium sensors are being developed (Rochefort and Konnerth 2008) and used to measure very short-lived calcium spikes (Grewe et al. 2010), and ultra-sensitive calcium indictors such as Cameleon-Nano are proving exceedingly useful (Horikawa et al. 2010). Two recent articles from Gilroy's lab have reviewed this topic in plants. One major review lists all the conventional fluorescence techniques for measuring Ca^{2+} (as well as ROS and pH) *in planta* (Swanson et al. 2011) and shows the emission spectra of various constructs including Cameleons. The more recent one (Choi et al. 2012) furnishes a very useful table listing different kinds of biosensors, their subcellular locale of assay, their targeting method, and cell types and plant species (primarily *Arabidopsis*) where they have been used (Table 5).

Hydraulic Signals

As mentioned earlier, when the xylem is massively perturbed by, for instance, flaming a leaf, it loses tension almost immediately, resulting in several signal-like phenomena. First, the loss of tension can be measured by changes in volume in the stem apex by the use of position-sensing transducers (Malone 1993; Stankovic et al. 1998). Second, the loss of tension results in

Table 5 Examples of the application of cytosolic and subcellularly targeted GFP-based biosensors for Ca^{2+}, pH, and reactive oxygen species (ROS) in plants

Biosensor	Subcellular locale	Targeting method	Cell types	Species	References
Ca^{2+}					
YC2.1	Cytosol	–	Root hair, root epidermis, guard cells, pollen tubes	*Arabidopsis*, *Medicago*, lily, tobacco	Miwa et al. (2006), Kosuta et al. (2008), Allen et al. (1999, 2001, 2002), Klusener et al. (2002), Hugouvieux et al. (2001), Capoen et al. (2011), and Watahiki et al. (2004)
	Nucleus	Nucleoplasmin fusion	Root hairs, pollen tubes	*Medicago*, tobacco	Sieberer et al. (2009) and Watahiki et al. (2004)
YC3.6	Cytosol	–	Stomatal guard cell, roots, root hairs, cotyledons, pollen tubes	*Arabidopsis*, lotus, tobacco	Weinl et al. (2008), Krebs et al. (2011), Iwano et al. (2009), and Monshausen et al. (2008, 2009, 2011)
	Plasma membrane	N-terminal fusion of YC3.6 with the LT16b protein	Roots, cotyledons	*Arabidopsis*	Krebs et al. (2011)
	Nucleus	NLS from SV40 large T protein	Roots, cotyledons	*Arabidopsis*	Krebs et al. (2011)
YC3.1	Cytosol	–	Pollen, stigmatic papillae	*Arabidopsis*, tobacco	Iwano et al. (2004), Certal et al. (2008), Michard et al. (2008, 2011), and Watahiki et al. (2004)
YC4.6	ER	Pumpkin 2S albumin signal peptide/HDEL ER retention signal	Pollen	*Arabidopsis*	Iwano et al. (2009)
D3cpv	Peroxisome	C-terminal KVK–SKL peptide	Roots, cotyledons	*Arabidopsis*	Costa et al. (2010)
	Tonoplast	N-terminus of CBL2	Roots, cotyledons	*Arabidopsis*	Krebs et al. (2011)
pH					
H148D	Cytosol	–	Roots, root hairs	*Arabidopsis*	Fasano et al. (2001) and Monshausen et al. (2007, 2009, 2011)
pHluorin	Cytosol	–	Roots, pollen tubes	*Arabidopsis*, tobacco	Gao et al. (2004), Moseyko and Feldman (2001), Certal et al. (2008), and Michard et al. (2008)
	Apoplast	Chitinase signal peptide	Roots	*Arabidopsis*	Gao et al. (2004)
Pt-GFP	Cytosol	–	Roots, leaves	*Arabidopsis*	Schulte et al. (2006)

(continued)

Table 5 (continued)

Biosensor	Subcellular locale	Targeting method	Cell types	Species	References
H_2O_2					
Hyper	Cytosol	–	Leaf epidermis, stomatal guard cells, suspension cell culture	*Arabidopsis*	Costa et al. (2010)
	Peroxisome	C-terminal KSRM peptide	Leaf epidermis, stomatal guard cells	*Arabidopsis*, tobacco	Costa et al. (2010)
Redox					
RoGFP1/2	Cytosol	–	Roots, leaves	*Arabidopsis*, tobacco	Jiang et al. (2006), Meyer et al. (2007), Rosenwasser et al. (2010), and Schwarzlander et al. (2009)
	Mitochondrion	First 87 amino acids of the tobacco β-ATPase	Roots, leaves	*Arabidopsis*, tobacco	Jiang et al. (2006), Rosenwasser et al. (2010), and Schwarzlander et al. (2008, 2009)
	ER	Chitinase targeting peptide/HDEL retention signal	Roots, tobacco leaf cells	*Arabidopsis*	Meyer et al. (2007) and Schwarzlander et al. (2008)
	Peroxisome	C-terminal SKL peptide	Leaves	*Arabidopsis*, tobacco	Rosenwasser et al. (2010) and Schwarzlander et al. (2008)
	Plastid	Transketolase target peptide	Leaves	*Arabidopsis*	Rosenwasser et al. (2010) and Schwarzlander et al. (2008)

Table 1 from Choi et al. (2012). Copyright The Plant Journal. Reproduced with permission

diminished water uptake through the roots, which can be measured by loss of water from a reservoir. Third, the transmitted loss of tension results in the local generation of a variation potential (which gets slower and smaller with distance from the wounded leaf). These three parameters have been measured simultaneously in the same plant (Davies et al. 1991).

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