Systemic signaling was investigated in both a dicot (Vicia faba) and a monocot (Hordeum vulgare) plant. Stimuli were applied to one leaf (S-leaf), and apoplastic responses were monitored on a distant leaf (target; T-leaf) with microelectrodes positioned in substomatal cavities of open stomata. Leaves that had been injured by cutting and to which a variety of cations were subsequently added caused voltage transients at the T-leaf, which are neither action potentials nor variation potentials: with respect to the cell interior, the initial polarity of these voltage transients is hyperpolarizing; they do not obey the all-or-none rule but depend on both the concentration and the type of substance added and propagate at 5 to 10 cm min\(^{-1}\). This response is thought to be due to the stimulation of the plasma membrane H\(^+\)-ATPase, a notion supported by the action of fusicoccin, which also causes such voltage transients to appear on the T-leaf, whereas orthovanadate prevents their propagation. Moreover, apoplastic ion flux analysis reveals that, in contrast to action or variation potentials, all of the investigated ion movements (Ca\(^{2+}\), K\(^+\), H\(^+\), and Cl\(^-\)) occur after the voltage change begins. We suggest that these wound-induced “system potentials” represent a new type of electrical long-distance signaling in higher plants.

Understanding systemic signaling in plants has long been recognized as a major scientific challenge. In principle, the systemic signaling induced by wounding and/or pathogen or herbivore attack may be realized by either chemical or electrical signals. Chemical signals have been shown to be involved in long-distance signaling, propagating likely from organ to organ either through the vascular system or as volatiles that are released into the atmosphere, carrying the message not only to organs within a plant but possibly to neighboring plants as well (Heil and Silva Bueno, 2007; Heil and Ton, 2008; Howe and Jander, 2008; Mithöfer et al., 2009). Other studies suggest that upon wounding, electrical signals may travel through phloem and/or xylem elements (Davies, 1987; Rhodes et al., 1996). Interestingly, such electrical signals have also been shown to affect systemic leaves, for example, by regulating genes (Graham et al., 1986; Wildon et al., 1992; Stankovic and Davies, 1997; Herde et al., 1998). Among other genes, proteinase inhibitor (Pin) and calmodulin mRNA have been up-regulated in tomato (Solanum lycopersicum) upon wounding and the application of heat stimuli (Stankovic and Davies, 1997). Plants that elicited no electrical signal did not accumulate Pin mRNA (Stankovic and Davies, 1997). In particular, the induction of Pin genes is striking because these proteinase inhibitors are induced upon insect herbivory as a defense reaction (Green and Ryan, 1972). Proteinase inhibitors either harm the attackers or simply prevent insects from feeding (Koiwa et al., 1997). Although, in principle, cellular reactions in plants have also been demonstrated to follow the release of electrical signals induced by heat, chilling, or electric voltage, to what extent such signals carry specific information in nonspecialized plants or organs is disputed.

In plants, a variety of electrical phenomena have been described and have to be considered as signal-transducing events. Whereas local voltage transients, due to system resistance, will vanish after a distance of a few millimeters and hence have no relevance for systemic signal transfer, action potentials (APs) and so-called variation potentials (VPs; for review, see Davies, 2006) may carry information over long distances from organ to organ. As demonstrated recently (Felle and Zimmermann, 2007), even if the channel activation was insufficient to trigger an AP, subthreshold depolarizations may have propagated along the stimulated leaf without proceeding to neighboring leaves, either because not enough channels were activated or because no signal-conducting connection existed between these leaves. Although such voltage changes suffer a decrement, information can be carried much farther than through simple voltage transients. APs and VPs are well documented in the literature (Davies, 2006). As electrical signals are fast, they may act as forerunners of slower traveling chemical signals, which might be located throughout the plant. For
instance, such signals might be released from injuries caused by herbivorous insects. Still, single APs, as “all-or-none” phenomena, likely do not contain much information regarding the kind of threat or stress that caused them; they may serve as general stress signals, however, which cause responses at the level of gene expression, primarily transcription but also translation (Wildon et al., 1992; Stankovic and Davies, 1997). Additional and specific information regarding the nature of the threat may come from chemical signals that are either transported within phloem and/or xylem elements or transferred through volatile substances (De Bruxelles and Roberts, 2001; Heil and Silva Bueno, 2007; Mithöfer et al., 2009).

So far, APs and VPs have been thought to be the only kind of electrical long-distance signals. In this study, we demonstrate a novel type of electrical signal that propagates systemically (i.e. from leaf to leaf), varies with intensity as well as with the nature of the original stimulus, and, therefore, is no AP. Since the initial direction of the signal is hyperpolarizing (with respect to the cell interior), it does not qualify for a VP in the classical sense (Stahlberg and Cosgrove, 1997). Thus, we propose the existence of a new electrical signal type, called “system potential” (SP). It operates by stimulating the plasma membrane H\(^+\)-ATPase, which may hold and transport information systemically within the whole plant or at least in parts of the plant. A detailed ion flux analysis is given. We demonstrate that this signal can be triggered by substances that are added to a leaf injured by cutting and transmitted systemically in Hordeum vulgare and Vicia faba as well as in a variety of other plants.

RESULTS

Release of SPs

Electrical signals were set off after mechanical injury to a leaf (cut) and the subsequent addition of inorganic cations (Ca\(^2+\), K\(^+\), Mg\(^{2+}\), or Na\(^+\)) or Glu to the site of injury. In accordance with the setup shown in Figure 1, the signals had to propagate first in the basipetal direction (S-leaf) and then in the acropetal direction through the stem to the T-leaf (20–25 cm in H. vulgare, 20–40 cm in V. faba) to be monitored with apoplastically positioned microprobes (Felle and Zimmermann, 2007). Without mechanically injuring the leaves, signals were either not released or weak. Cutting alone caused an immediate fast transient voltage jump of a few millivolts on the T-leaf but had no obvious significant effect thereafter. As shown in Figure 2, after wounding and stimulation with Glu, we found two clearly different voltage responses in the T-leaf apoplast: APs with the usual polarity and/or voltage transients in the inverse direction. A similar chain of events occurs due to other stimuli (e.g. inorganic ions). As demonstrated recently (Felle and Zimmermann, 2007), inorganic cations (Ca\(^2+\), K\(^+\), Na\(^+\), and Mg\(^{2+}\)) applied to the cut leaf may or may not trigger an AP that propagates systemically from leaf to leaf. When it does not, a voltage transient like the one shown in Figure 2B appears at the T-leaf. This response, called SP, is both substrate and concentration dependent (Fig. 3). Of the ions tested, Ca\(^{2+}\) proved to be the most effective. Although SPs could be recorded with Ca\(^{2+}\) concentrations at 1 mM (kinetics not shown), mostly higher concentrations were used to obtain the best responses possible. Typically, the SPs propagated at 5 to 10 cm min\(^{-1}\). Since an SP had to propagate first basipetally and then acropetally to reach the systemic leaf, a direct effect of the applied ion (e.g. through long-distance transport) can be excluded. Moreover, the responses to the different cations (Fig. 3A) clearly indicate that the anion (Cl\(^-\)) is not involved. Mannitol (50 mM), a nondepolarizing agent, had no effect (data not shown). Apart from H. vulgare and V. faba, SPs were also recorded on Nicotiana tabacum, Phaseolus lunatus, and Zea mays (data not shown).

Extracellular Versus Intracellular Responses

To prove that apoplastic and intracellular voltage changes correspond, control experiments were performed in which the intracellular and extracellular responses to Ca\(^{2+}\) were measured simultaneously (Fig. 4). The observation that the extracellular voltage change considerably exceeds the change in membrane potential is based on the fact that the electrical resistances of apoplast and symplast differ.

The Effects of H\(^+\) Pump Stimulation or Inhibition

Fusicoccin (FC) is a well-known fungal toxin that stimulates H\(^+\) extrusion and hyperpolarizes the plasma membrane of plants (Marre, 1979). When added to the S-leaf, after due time 1 \(\mu\)M FC causes a hyperpolarizing...
response at the T-leaf similar to the one detected with the cations (Fig. 5B). An SP-initiating Ca\(^{2+}\) treatment applied to the same leaf after an FC treatment no longer had any effect (kinetics not shown). Orthovanadate, a P-type ATPase inhibitor, generally prevented the propagation of SPs (Fig. 5, B and C). After orthovanadate was injected into the apoplast of the target leaf in *H. vulgare* (Fig. 5A) and the solution had been absorbed by the affected tissue, tests were carried out. Electrodes were positioned roughly 2 cm before and 2 cm behind the orthovanadate-treated area. Noninvasive control “light/dark” tests proved the full responsiveness of the tissue at both electrodes. Figure 5B shows the FC response at electrode 1 and the missing response behind the orthovanadate-treated area at electrode 2. SPs generated by Ca\(^{2+}\) were likewise stopped from propagating after orthovanadate treatment at the S-leaf (data not shown). Infiltrating the leaf with artificial apoplastic fluid (AAF; 2 mM KCl and 0.1 mM CaCl\(_2\), pH 5) used as a control did not stop the propagation of the signal but did weaken it (control AAF). Adding orthovanadate to the wounded region before cation treatment prevented the release of an SP (kinetics not shown) but, after due propagation time, massively hyperpolarized the apoplastic potential (depolarized the membrane potential) on the T-leaf about 30 cm away from the stimulus site (Fig. 5C). To test whether the orthovanadate could have been transported during the elapsed time from the site of stimulation to the receiving electrode, 25 mM KCl was added to the wounded region and a K\(^{+}\) selective microelectrode was placed at the neighboring leaf to pick up K\(^{+}\) shifts. As shown in Figure 5C, in the first 30 min, no increase of apoplastic [K\(^{+}\)] was recorded.

**Ion Movements**

As recently demonstrated (Felle and Zimmermann, 2007), release and propagation of APs are causally linked to ion movements, due to the selective activation of channels. Since SPs are essentially hyperpolarizing events, most likely generated and transmitted by H\(^{+}\) pump activation, it was of interest whether this would show up in the ion movements as well. Figure 6 shows the ion movements that occur during their pertinent voltage changes (SPs); these movements have been aligned to point out the temporal sequence of the ion movements. Apoplastic Ca\(^{2+}\), K\(^{+}\), and H\(^{+}\) activities decreased; only Cl\(^{-}\) increased. All of the ion movements were transient, and none of the ion movements occurred before the voltage changes.

**DISCUSSION**

In *H. vulgare* and *V. faba* as well as in other plants such as *N. tabacum*, *P. lunatus*, and *Z. mays* (data not shown), we demonstrate a new kind of electrical long-distance signal that propagates systemically, the SP. As shown in Figures 2 and 3, in combination with mechanical wounding, SP signals are triggered by inorganic cations such as Ca\(^{2+}\), Na\(^{+}\), and Mg\(^{2+}\) or organic compounds such as Glu. Obviously, SPs are neither VPs nor APs. In Table I, the most basic characteristics of the three kinds of signals are compared: unlike the primary polarity in VPs and APs, the...
primary polarity of SPs is reversed; moreover, SPs do not obey all-or-none conditions and are not caused by a hydraulic pressure surge or activation of ion channels. As the amplitude of the SPs is modified by the concentration as well as by the nature of a substance, the level of information potentially transferred by SPs should be higher than that transferred by a single AP, which cannot be modulated in amplitude.

SPs Are Due to a Stimulation of the H⁺-ATPase

What is the nature of these SPs and how are they generated? In a recent paper, we demonstrated that in *H. vulgare*, APs were triggered by a variety of substances like inorganic ions, Glu, et cetera (Felle and Zimmermann, 2007). The release of a systemic AP was shown to be critical: when the stimulus was too weak to activate enough channels or the system resistance around the nodi was too high, APs were either not released at all or did not propagate to the T-leaf. However, even weak stimuli are sufficient to generate an SP, providing that a substantial depolarization occurs. To explain this, one has to recall that the two main functions of a plasma membrane H⁺-ATPase are to generate a transmembrane electrochemical H⁺ gradient and to maintain a constant H⁺ turnover, both of which are necessary for transmembrane transport processes. The undisturbed membrane establishes a dynamic equilibrium (i.e. a stable membrane potential), transmembrane ion gradients, and ion fluxes. As soon as this equilibrium is disturbed, for example, by a depolarization of any kind, the H⁺-ATPase will re-
respond with increased activity in order to restore the resting state of the membrane potential. This effect, a (partial or total) repolarization, is quite common and follows the addition of cations or cotransported substrates to the external phase of the plasma membrane. Such a scenario apparently happens in the wounded leaf region after cations are added. Clearly, the initial depolarization will suffer a decrement and vanish within a short distance (millimeters) from the origin, while the H+-ATPase stimulation sustains yields of hyperpolarization (or apoplastic depolarization); this hyperpolarization obviously propagates and can be picked up intracellularly as well as within the apoplast of the target leaf. On the other hand, a primary hyperpolarization following pump stimulation by FC does not have to be transformed but propagates as it is. Actually, SPs may be equivalent to the late recovery section of an AP (Fig. 2), which has been demonstrated to depend on pump activity (Felle and Zimmermann, 2007). Therefore, we suggest that the phenomena shown here reflect nothing but hyperpolarization caused by a temporary stimulation of the plasma membrane H+-ATPase(s). The observation that an FC-induced hyperpolarization actually propagates from one leaf to another strongly supports our hypothesis, which is backed up by the following observations: (1) after FC treatment, other agents no longer trigger SPs; (2) orthovanadate, a potent inhibitor of P-type H+-ATPases, prevents the propagation of SPs; and (3) the depolarization induced at the site of orthovanadate addition is carried systemically from one leaf to another, indicating that an inhibition of the pump is also carried systemically. The effects shown here cannot be explained by mass transport, which would be far more time consuming, and the observation that an increase in K+ (used as transport test substance) at the stimulus site is not carried to the measuring site on the T-leaf supports this notion (Fig. 5C). Evidently, SPs are self-propagating systemic events, which, unlike APs, do not need the mediation of channel activation. Because of the low area density for channels (1–3 per \( \text{mm}^2 \)), the release of an AP requires a channel to indirectly communicate through a substantial voltage change of a certain (probably critical) membrane area and the subsequent activation of voltage-gated channels or ligand activation (e.g. Glu). In contrast, propagation by pumps is conceivable by direct protein-protein interactions and activation transfer by molecular contact, because of their much higher area density (approximately 1,000 per \( \text{mm}^2 \)). Moreover, in particular for the H+-ATPase in plant plasma membranes, oligomerization has been demonstrated upon activation that was mediated by 14-3-3 proteins (Ottmann et al., 2007).

### The Chain of Events

Whereas with APs a Ca\(^{2+}\) influx clearly precedes the rapid voltage response and anion efflux is responsible

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>APs</th>
<th>VPs</th>
<th>SPs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Induction</td>
<td>Voltage threshold</td>
<td>Rapid turgor increase</td>
<td>Plasma membrane depolarization</td>
</tr>
<tr>
<td>Propagation</td>
<td>Self-propagating</td>
<td>Non-self-propagating</td>
<td>Self-propagating</td>
</tr>
<tr>
<td>Rate</td>
<td>20–400 cm min(^{-1})</td>
<td>10 s to several minutes</td>
<td>5–10 cm min(^{-1})</td>
</tr>
<tr>
<td>Mechanism</td>
<td>Activation of ion channels ((\text{Ca}^{2+}, \text{Cl}^{-}, \text{K}^+))</td>
<td>Inactivation of the H(^{+}) pump</td>
<td>Activation of the H(^{+}) pump</td>
</tr>
<tr>
<td>Ion movements and (\Delta V)</td>
<td>Ca(^{2+}) triggers Cl(^{-}) efflux and (\Delta V)</td>
<td>Causalities unclear</td>
<td>Ion movements follow voltage</td>
</tr>
<tr>
<td>Direction</td>
<td>Depolarization</td>
<td>Depolarization</td>
<td>Hyperpolarization</td>
</tr>
<tr>
<td>Duration of initial voltage change</td>
<td>&lt;20 s</td>
<td>10 s to several minutes</td>
<td>8–12 min</td>
</tr>
<tr>
<td>Signal</td>
<td>All or none</td>
<td>Graded signals of variable size</td>
<td>Signals depend on stimulus</td>
</tr>
</tbody>
</table>
for the typical AP “breakthrough,” the ion fluxes accompanying the SP occur not prior to the voltage response but after its onset (Fig. 6), indicating that these ion movements are a result of the SP rather than its cause. This interpretation is also strongly supported by the observation that the direction of $K^+$, $Ca^{2+}$, and $Cl^-$ fluxes clearly follows the voltage changes (i.e. changes in driving force). The development of apoplastic pH appears to be the only characteristic that APs, VPs, and SPs have in common, namely an alkalinization. It has been demonstrated that apoplastic pH increase is a typical stress response to drought (Wilkinson, 1999), salt stress, low temperature, and fungal attack (Felle et al., 2005), and oxygen shortage (Felle, 2006). Despite the apparently logical assumption that pump activation should acidify the apoplastic pH and alkalize the cytoplasm, there is ample evidence in the literature that pump activity and pH (changes) are not necessarily related. The observation that the apoplastic pH increases in the recovery phase of APs, when the pump actively repolarizes or even hyperpolarizes the plasma membrane (Felle and Zimmermann, 2007), clearly shows that pump activity and apoplastic pH or changes thereof are not related. Apart from being buffered by weak acids/bases or being part of biochemical reactions that produce/ consume $H^+$, pH is a general mediator and regulator of membrane transport that also involves other ions than $H^+$. Acid base chemistry (Stewart, 1983) predicts that a pH change within a given compartment is not dependent on a transmembrane $H^+$ displacement but on a change of the strong ion ratio. FC, for example, which undoubtedly stimulates the plasma membrane $H^+$ pump and $H^+$ extrusion, may lead to external alkalinization (Ullrich et al., 1991) and may acidify the cytoplasm (Bertl and Felle, 1985), or it may hardly affect the pH of either side of the membrane (Ullrich and Novacky, 1990). Here, we observe that an insignificant initial pH decrease is followed by a substantial transient pH increase while the voltage recovers. The apoplastic alkalinization observed here may have several causes. (1) Due to the known poor selectivity of anion channels (Hedrich et al., 1994; Schmidt and Schroeder, 1994), the efflux of $Cl^-$ is accompanied by the efflux of a variety of organic acids; as soon as these enter the acidic apoplast, protons are bound and cause a pH increase. (2) Due to the hyperpolarization, the driving force for $H^+$ cotransport is increased, which could drain the apoplast of $H^+$ to some extent. (3) Cell walls contain high concentrations of uronic acids with $pK$ values similar to that of polygalacturonic acid. Thus, either cations of the apoplast are reversibly retained as free hydrated ions or they become immobilized. An activity decrease of cations (Fig. 6) means more free negative charges that can be occupied by $H^+$, which will increase the apoplastic pH, as demonstrated (Felle, 1998). Thus, the somewhat unexpected pH response would not contradict our pump hypothesis.

The Novelty of SPs

Hyperpolarization during long-distance signaling has been reported before. (1) Eschrich et al. (1988) investigated the transmission of electric signals in sieve tubes of zucchini (Cucurbita pepo) and observed AP-like depolarizations induced by the addition of 100 mM Suc at the petiole, which was picked up after 10 to 40 s as hyperpolarization at the fruit (40 cm away). Although no definite interpretation was given, it is possible that the phenomenon is similar to what we are describing here. On the other hand, the observation that a hyperpolarization at the petiole turned into a depolarization at the fruit would make this interpretation unlikely. (2) Fromm and Eschrich (1993) showed that 2 mM MgSO$_4$ added to the roots of willow (Salix viminalis) causes an immediate rapid and transient hyperpolarization, which is transmitted without decrement at 5 cm s$^{-1}$ to the leaf mesophyll. Since the velocity of these transmissions, their duration, and their lack of decrement were very different from what we found, we suggest that these AP-like phenomena have nothing in common with the SPs. (3) Fromm et al. (1997), testing the effects of phytohormones on the endogenous current in willow roots, observed an abscisic acid-induced hyperpolarization that was transferred from the root to the tip. Lautner et al. (2005) describe chilling-induced hyperpolarizations in poplar (Populus species) leaves, which propagated basipetally. In both reports, the authors presented evidence that hyperpolarizations were caused by $K^+$ channel activation; this is not comparable to the SPs, where $K^+$ fluxes were shown to be the result and not the cause of the voltage changes (Fig. 6).

Again, this study is not about long-distance signaling in highly specialized plants or organs; it is directed toward the ordinary plant that encounters stresses and hazards to which it must respond quickly and appropriately. In such plants, APs and VPs have so far been considered the only relevant electrical long-distance signals, a view that ought to be reconsidered. In Table I, the most essential characteristics of APs, VPs, and SPs are compared with each other, showing that SPs do not have much in common with APs or VPs. Whereas due to their all-or-none characteristics, APs do not carry much information with respect to the nature or intensity of the triggering stimulus, SPs are modulated in amplitude as well as in their independent ion fluxes, from which the plant or the affected organ may be able to gain information about the nature and intensity of the threat or injury. Thus, the SPs described here are a basic kind of self-propagating signal that may occur regularly when the membrane potential is shifted considerably from its set value through the combination of an injury and a chemical stimulus, the result of which is challenged by $H^+$ pump activity changes. This way, the systemic organ encounters a broad spectrum of information regarding the kind of disturbance suffered some distance away. The information transferred lies not only in the voltage
change but also in the changes in ion activities on both sides of the membrane: pH changes will alter enzyme activities and gene activation, K⁺ changes will cause water flow and cell turgor, and Ca²⁺ influx will increase cytosolic free Ca²⁺ and modulate signal chains.

Whether SPs are in fact a main electric signal transmission following, for instance, herbivore attack in order to initiate systemic defense or priming is the focus of our ongoing research. Preliminary data suggest that this likely is the case.

**MATERIALS AND METHODS**

**Plant Material**

Plants of *Vicia faba* and *Horddeum vulgare* ‘Ingrid’ (Deutsche Saatveredelung) were grown from seed in a plastic pot under a 12-h/12-h light/dark regime at 20°C to 25°C in a greenhouse. Intact 40- to 50-cm-long *V. faba* or three- to four-leaved *H. vulgare* plants were used throughout the experiments.

**Recording Apoplastic Voltage**

Whole *V. faba* plants or *H. vulgare* plants were mounted on a cuvette inside a Faraday cage. As described earlier (Felle et al., 2000; Felle and Zimmermann, 2007), the target leaves were tightly fixed on a Plexiglas plate with a double adhesive tape to prevent movement during the measurements. The leaves were illuminated with a cold light lamp (Leica; KL1500; Wetzlar) to induce stomatal opening. Under optical control (microscope) using a 20× long-distance objective, two or three blunt electrodes (tip diameter about 5 µm; filled with 0.5 M KCl/agar) were positioned at an angle of 40° to 50° in substomatal cavities of neighboring open stomata. The earth electrode (filled with 0.5 M KCl) was placed at the cut tip of this leaf, submerged in a solution comprising 10 mM KCl, 1 mM CaCl₂, and 1 mM MES + Tris, and mixed to pH 5. Electrodes were connected with a high-impedance amplifier (World Precision Instruments; FD223); kinetics were recorded by a pen chart recorder (Linseis; L2200). As soon as the tips of the electrodes came into contact with the apoplastic fluid, the electrical circuit was closed. The voltages given depend on the distance and the apoplastic network resistance between the voltage and the earth electrodes. Membrane potential measurements were carried out with sharp tips (0.5 µm) by inserting them into a mesophyll cell.

To test nonbiotic systemic responses, a leaf (S-leaf) was cut with scissors and the test solution (stimulus) was immediately applied; the response to this treatment was monitored with two or three electrodes on a different leaf (T-leaf; Fig. 1). The solutions tested were KCl, CaCl₂, MgCl₂, NaCl, glu, FC, and the test solution (stimulus) was immediately applied; the response to this stimulus follows, for instance, herbivore attack in order to initiate systemic defense or priming is the focus of our ongoing research. Preliminary data suggest that this likely is the case.

**Conventions**

With an intracellular recording, a depolarization of the plasma membrane occurs when the cell interior becomes less negative, whereas for an apoplastic recording, the reverse argument holds true. To avoid confusion, throughout this article we follow the convention and call an apoplastic hyperpolarization a depolarization. Since apoplastic voltage can be influenced by a variety of processes and, therefore, unlike a membrane potential is not clearly defined, we give no absolute values, just the polarity (+) together with relative voltage.

**LITERATURE CITED**


(1994) Malate-sensitive anion channels enable guard cells to sense changes in the ambient CO₂ concentration. Plant J 6: 741–748


