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Review Article

Electropenetrography: A New Diagnostic Technology for Study of Feeding Behavior of Piercing-Sucking Insects†

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Abstract

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Studying the feeding, plant damage, and transmission (i.e., acquisition, retention, and inoculation) of plant pathogens caused by hemipteran insect pests has always been a challenge. This is primarily because their specialized, piercing-sucking mouthparts, the stylets, perform probing/penetrating action into opaque plant tissues in which the stylets cannot be directly visualized. This challenge was overcome by a technological revolution over 50 years ago; the invention of electropenetrography, or electrical penetration graph (EPG) monitoring, the most rigorous method to identify feeding behaviors of hemipteran crop pests. Today, EPG is used in three main areas for the development of novel integrated pest management (IPM) tactics for hemipteran pests. Firstly, in cases where the fundamental mechanisms of feeding damage or transmission of a plant pathogen are unknown, EPG is instrumental in identifying such mechanisms. Secondly, once the feeding-related causes of damage or pathogen transmission are understood, EPG can be used to demonstrate the effects of insecticides, antifeedants, or other chemical compounds on specific feeding behaviors responsible for the damage or transmission. Thirdly, EPG can similarly identify the effects of resistant vs. susceptible varieties of crop plants, including transgenic plants genetically engineered to express biopesticides. The purpose of this paper is to review: (1) principles and history of EPG, especially development of the new and improved, third-generation AC-DC monitor, (2) what waveforms and types of information can be gained via EPG, using aphid pests as a model system, and (3) how EPG can be applied to the special needs of Taiwanese agriculture, especially for some species of aphids of economic significance in Taiwan. For the first time in print, our review describes and discusses in details the information of all three types of EPG monitors used for researches on aphids.

Key words: Electrical penetration graph (EPG), Hemiptera, Feeding behavior.

INTRODUCTION

Studying the feeding, plant damage, and (especially) transmission (i.e., acquisition, retention, and inoculation) of plant pathogens caused by hemipteran insect pests has always been a challenge. This is primarily because their specialized, piercing-sucking mouthparts, the stylets, perform probing/penetration into opaque plant tissues in which the stylets can-

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not be directly visualized. Early studies of hemipteran feeding were forced (by the technical limitations of their time) to study snapshots of feeding, after the fact. For example, one could view deposits of hardened saliva *in planta* left behind after feeding activities had ceased (Leopold *et al*. 2003), in essence, frozen in time. Or, one could quantify collections of excretory droplets (Dugravot *et al*. 2008) or document transmission of plant pathogens from long-past stylet probes (Purcell *et al*. 1979; Almeida & Purcell 2006). However, a researcher could not directly study or quantify hemipteran feeding in real time, as it was occurring, until the invention of a revolutionary technology over 50 years ago. That technology is electropenetrography, also known as electrical penetration graph (both names abbreviated as EPG) monitoring of insect feeding (Walker 2000; Almeida & Backus 2004; Backus *et al*. 2005). Most advances in understanding hemipteran feeding behavior have been made possible by use of EPG over the last 50 years.

Today, EPG is used in three main areas for the development of novel integrated pest management (IPM) tactics for hemipteran pests, especially vectors of plant pathogens. Firstly, in cases where the fundamental mechanisms of feeding damage or transmission of a plant pathogen are unknown, EPG is instrumental in elucidating such information. Secondly, once such mechanisms are understood, EPG can be used to demonstrate the effects of insecticides, antifeedants, or other chemical compounds on specific feeding behaviors responsible for damage or transmission. Thirdly, EPG can similarly identify the effects of resistant vs. susceptible varieties of crop plants, including transgenic plants genetically engineered to express biopesticides. Rapid computerized analysis of EPG data can provide quantitative comparisons of, for example, the responses of vectors to resistant and susceptible plants. A researcher can then predict whether a pathogen will be transmitted from/to a putatively resistant host plant, leading to a novel mechanism for host plant resistance.

The purpose of this paper is to review: (1) principles and history of EPG, especially the development of the new and improved, third-generation AC-DC monitor, (2) what waveforms and types of information can be gained via EPG, using aphid pests as a model system, and (3) how EPG can be applied to the special needs of Taiwanese agriculture, especially for seven species of aphids of economic significance. For the first time in print, our review describes and discusses in details the information of all three types of EPG monitors used for research on aphids. Future papers will review the use of EPG in studies of leafhopper, planthopper, psyllid, and whitefly feeding in relation to Taiwanese agriculture. Some of the narrative herein on EPG principles is excerpted and/or adapted from (Backus in press), to which the reader is referred to for more information.

HISTORY AND PRINCIPLES OF ELECTROPENETROGRAPHY

Basic principles. As diagrammed in Fig. 1A, the insect is made a part of an electrical circuit by attaching a thin (~10–60 μm) gold wire to its dorsal surface with conductive glue or paint, then connecting the insect to the input of a head stage amplifier attached to a monitor that also electrifies the plant (Vs to the electrode in the plant soil, Fig. 1). When the insect's stylets probe the plant tissues, fluids in the stylet food and salivary canals ionically conduct the electrical signal through the insect to the monitor, where it is amplified (secondary signal processing circuit, Fig. 1) and outputted to a computerized digital display (not depicted in Fig. 1). Variable biopotentials (i.e., biological voltages or emf [see below] in Fig. 1B) and/or electrical resistances to fluid flow (Ra [see below] in Fig. 1B) generated by the insect-plant interface instantaneously transform

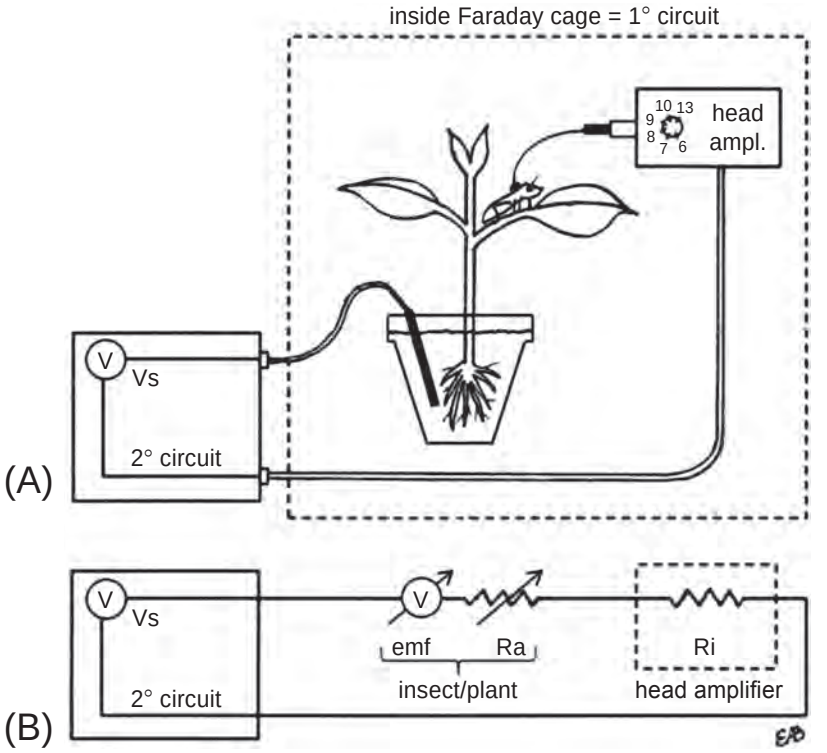

Fig. 1. Diagrammatic representation of the primary (1°) circuit of an EPG monitor. (A) Realistic model of the plant and insect. (B) Electronic block diagram of primary circuit, including variable biopotentials (emf) and variable resistance (Ra). 2° = secondary circuit (i.e., signal processing circuitry); head ampl. = head stage amplifier; emf = electromotive force (biopotential); Ra = insect (e.g., aphid) resistance; Ri = input impedance of the head amplifier; Vs = source voltage. (Derived from an original drawing by G. Walker, in Walker 2000. Reprinted with permission of the American Phytopathological Society)

the constant applied signal into a fluctuating-voltage output signal that is graphed over time as a waveform.

History and monitor designs. First developed in the late 1950s to early 1960s, EPG has advanced over the last 50 years along with the revolution in electronics. The earliest, first-generation EPG monitors, developed by McLean and Kinsey, used technology typical of the time, i.e., glass-tube amplifiers in the late 1950s, later evolving into early solid-state transistors by the 1960s (McLean & Kinsey 1964). They also used AC (alternating current) applied signal, and a low amplifier sensitivity or input impedance (Ri) of 106 Ohms (McLean & Weigt 1968), in a design inspired by radio technology, with which McLean was familiar. These early 'AC monitors' with low Ri outputted signals that were caused primarily by electrical resistance to/conductance of ionic charges carried in fluids (e.g. saliva, plant fluids) passing through the stylets (modeled as a variable resistor, Ra, in Fig. 1B) (Walker 2000). Thus, the earliest AC monitors detected for the first time, in real time, vital information such as beginning and termination of stylet probing, saliva secretion and salivary sheath formation (today termed pathway activities), stylet movements such as extension, retraction, and partial stylet withdrawal, and stylet contact with vascular tissues such as phloem and xylem (Backus *et al*. 2005). Some AC monitor designs actually could detect a small fraction of the biopotentials (voltages) in the circuit (emf,

defined below) (Backus *et al*. 2000); however, practically speaking, AC monitors outputted virtually no emf, due primarily to low Ri level and the then-prevalent use of slow-response strip chart recorders, but also secondarily to bandpass filters found in certain AC monitor designs (Backus *et al*. 2000; Tjallingii 2000).

By the late 1970s, electronics had been revolutionized with improved solid-state transistor technology (by then termed operational amplifiers, or op amps), so that more sophisticated amplifiers and recording devices were available and affordable. The design of the second-generation electropenetrography (termed 'DC monitor' or 'DC system') was introduced by Schaefers (1966), but greatly improved by Tjallingii (1978). Tjallingii used op amps in simple printed circuits, DC (direct current) applied signal, Faraday cages to control noise, FM tape recorders or rapid-response strip chart recorders as output devices, and (most importantly) higher amplifier sensitivity (Ri of either 109 or 1013 Ohms) (Tjallingii 1978, 1985a). Tjallingii's elegant analyses (Tjallingii 1978, 1985a) also established the modern theoretical understanding for EPG science by introducing the concepts of the R (or resistance) component (Ra, described above) and the electromotive force, emf (nearly synonymous with biological voltage or biopotential) component, blended together in the output signal (Walker 2000). The R and emf components are also termed electrical origins of a waveform, and are the foundation of modern EPG science.

There are two known mechanisms underlying the emf component in the plant or plant-insect interface. The first is disruption of the plant cell membrane's charge separation between external and internal cell environments, by stylet tips breaking living plant cell membranes. Such membrane breakages lead to two electrical effects on waveform output: (1) sudden voltage drops as the stylets puncture a membrane, and (2) positive or negative voltage levels that indicate extracellular (apoplastic) vs. intracellular (symplastic) stylet tip positions, respectively. The second biopotential mechanism is streaming potentials, i.e., tiny voltages developed by charge separation that occurs nearly instantaneously in ionic fluids rapidly moving through thin capillary tubes, such as the stylet food and salivary canals (Walker 2000). Streaming potentials cause regular-frequency waveforms that result from rhythmic pumping of muscles (Walker 2000), such as the cibarial dilator muscles controlling uptake of fluid into the functional foregut (Dugravot *et al*. 2008). Increased Ri level in the DC monitor (compared with AC monitor designs) made it possible to detect both R components (previously detected by the AC monitor) and additional emf components in the EPG output waveform. Identifying the electrical origin(s) of a waveform greatly aids in defining its biological meaning.

Another valuable theory of Tjallingii was the sigmoidal emf responsiveness curve (Tjallingii 1978, 1985a), later renamed the R/emf responsiveness curve (Backus & Bennett 2009; Backus in press). The curve is produced when the proportion (0–100%) of emf in an insect's total EPG output signal (Walker 2000) is graphed in relation to Ri level (on a scale of 106 to 1013 Ohms) (Backus & Bennett 2009). The lower the Ri level, the smaller the proportion of the total signal that consists of emf (Fig. 2). Because the ratio of R/emf is reciprocal within the total EPG signal, the smaller the emf proportion, the larger the R proportion (Backus *et al*. 2000; Walker 2000; Backus & Bennett 2009). In addition, the position of the R/emf responsiveness curve with respect to Ri can shift with the size of the insect being recorded [Fig. 2; compare small aphids vs. small leafhoppers vs. large sharpshooter leafhoppers (Cicadellidae: Cicadellinae)]. Generally, the larger an insect's body (i.e., with larger-diameter food and salivary canals, therefore greater ionic conductivity), the more the responsiveness curve shifts towards the left on the chart (Fig. 2). Thus, lower Ri levels allow detection

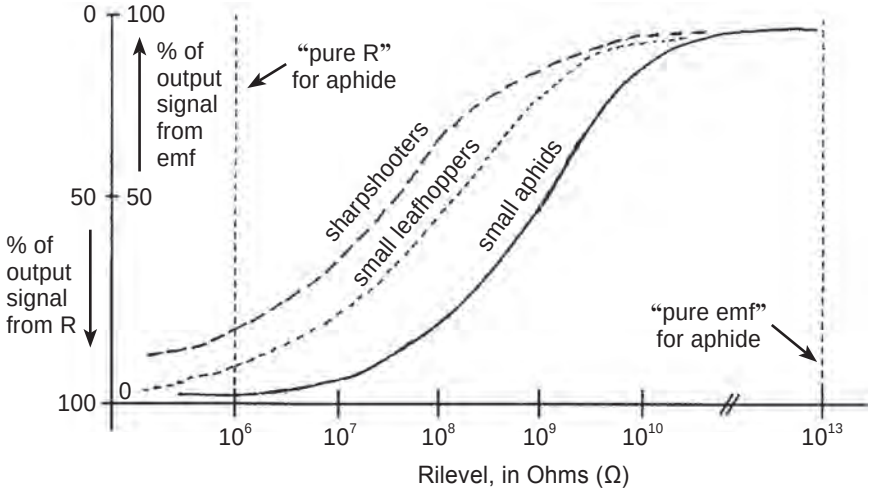

Fig. 2. Theoretical R/emf responsiveness curves for selected example insects. See text for summary, and (Backus & Bennett 2009) for more detailed explanation of the theory. (Reprinted with permission of the American Phytopathological Society)

of more emf in the signal of large hemipterans like sharpshooter leafhoppers, compared with smaller species like aphids. The Ri level that represents the 50:50 R/emf balance point will detect the maximum number of waveforms from both electrical origins, and thus present the best representation of all possible probing behaviors. Tjallingii chose an intermediate Ri level of 109 Ohms for his (DC monitor) design, to balance R and emf components for small aphids. Thus, the R/emf responsiveness curve explains why an AC monitor (Ri 106 Ohms) detected almost no emf component in aphid probing (Backus & Bennett 2009), but slightly more in waveforms of sharpshooters and other large insects (Backus *et al*. 2005).

By the mid-1990s, all EPG researchers were using computerized analog-to-digital waveform display, greatly improving waveform fine-structure detail from both AC and DC recordings (Reese *et al*. 1994). Also by that time, EPG had gradually become specialized so that AC monitors were used primarily for medium-to-large insects such as leafhoppers, planthoppers, and other auchenorrhynchans, while DC monitors were used for smaller insects, especially aphids, whiteflies, thrips, psyllids, and other sternorrynchans (Backus 1994). Such specialization lasted until AC monitors were no longer manufactured (about 2,000); since then, most research has used DC monitors until recently. In retrospect, the R/emf responsiveness curve explains part of the specialization of AC versus DC recordings for differently-sized insects, because signals from small insects like sternorrhynchans would contain both R and emf at Ri 109 Ohms, while recordings of larger insects like auchenorrhynchans would contain both components at lower input impedance (Ri 106 Ohms). However, another reason for past specialization may be due to a difference in tolerance to type of applied signal. Although such differential tolerance is poorly known and an active area of research, anecdotal observations suggest that large leafhoppers like sharpshooters seem to tolerate AC better than DC at low Ri levels, while aphids may tolerate DC better (Backus *unpub. data*). This is important because many R-dominant waveforms can only be detected with rather high applied voltages (hundreds of mV) combined with low Ri (Ri 10^6 or 10^7 Ohms). When high voltages are used, the insect's physiology must tolerate such high current densities. For

some reason (perhaps related to intercellular versus intracellular style of feeding, see below), different insects seem to tolerate DC or AC better at low Ri combined with high voltages.

To increase flexibility for all types and sizes of insects, a third-generation, 'universal' EPG monitor (termed AC-DC) was developed by Backus and Bennett (Backus & Bennett 2009). It provides selectable Ri levels of 106–1,010 plus 1,013 Ohms, choice of AC or DC applied signal, and modern, up-to-date electronics such as instrumentation-quality op amps on standardized, commercially-manufactured printed circuit boards. This instrument removes artifactual voltages and other problems of older electronics (Backus *et al*. 2000; Backus & Bennett 2009), and has controls that are highly sensitive and scalable. Although termed 'AC-DC', the 'universal' instrument is not the same as the old AC monitor technology. The AC-DC monitor was designed to incorporate all functions of the second-generation DC monitor, including retaining emf despite making AC applied signal available (for use with insects that don't tolerate DC at high voltages). For example, the AC signal processing circuit no longer has highpass/bandpass filters, coupling capacitors, log amplifiers, or other components now understood to remove emf from the output signal (Backus *et al*. 2000; Tjallingii 2000; Backus & Bennett 2009). Although rectification is still performed for AC signal processing, a voltage offset knob allows the user to remove the waveform fold-over caused by rectification of negative-going waveforms, restoring perfect waveform fidelity to output of the DC monitor. In addition, the third-generation monitor expands EPG technology to include the first-ever, user-defined choice of Ri levels, very high amplification capabilities, and precise reproducibility of settings with high-resolution dials for gain and applied voltage control, allowing settings to be exactly reproduced among recordings (Backus & Bennett 2009).

As a result of the above design innovations, the AC-DC monitor combines all the advantages of both previous generations of EPG monitor, with virtually none of the disadvantages. Thus, a researcher can precisely tailor the monitor settings to the specific needs of each insect species recorded, allowing characterization of a 'waveform library' of output signals at different Ri levels, more accurately and correctly defining emf- versus R-components (Backus *et al*. 2013; Pearson *et al*. 2014).

Using the AC-DC monitor, it was found that there were no differences in appearance of aphid waveforms based on type of applied signal *per se*, i.e., AC versus DC. However, large differences occurred based on Ri level, as predicted by the R/emf responsiveness curve (Backus & Bennett 2009). These results support the success of the design goals for this monitor. In a similar manner, differences in amplitude among waveforms can be used to demonstrate the effects of the R/emf responsiveness curve, to determine electrical origins of waveforms by changing the Ri level.

Principles underlying biological meanings of EPG waveforms. The biological meanings of EPG waveforms are defined by correlation with: (1) stylet tip locations in the plant (e.g. vascular versus non-vascular tissue termini), via histology of salivary sheaths, cut stylets in probed plant tissues, and/or fluid exudation of cut stylets, (2) intricate stylet activities (e.g., stylet movements, salivation, etc.) performed by the insect, via observation in transparent artificial diets, and other methods, and (3) electrical characteristics of the waveforms, some of which have been explained above (Walker 2000).

Additional electrical characteristics of waveforms include those that reveal stylet depth. This can occur in two ways, depending upon Ri level. Waveforms recorded at lower Ri level (106 or 107 Ohms, depending on species) are usually positive-going, and the position of the waveform above 0 V baseline

(termed voltage level) is correlated with stylet depth (see figures in Backus *in press*). In other words, the voltage level rises or falls as the stylets are partially withdrawn or pushed more deeply into the plant tissue, respectively (Jiang & Walker 2001; Backus *et al*. 2005). Thus, stylet depth in the plant is a strong R component. At higher Ri settings (10^9–10^{13} Ohms), waveforms usually become both positive- and negative-going (Figs. 3A and 3B), and the biological meaning of voltage level shifts so that it reflects the electrical charge immediately surrounding the stylets tips; thus plant electrical charges internal or external to cells create a biopotential, i.e., an emf component. Negative voltage level (termed 'intracellular' level in the DC EPG literature) (Walker 2000) denotes symplastic space (i.e., inside living cells whose charge separation between external and internal environment is maintained by an intact plasma lemma that is either not disrupted by stylet penetration or sealed by sheath saliva). Positive voltage level (termed 'extracellular') denotes apoplastic space in the plant (i.e., intercellular spaces, interior of cell walls between living cell membranes, mature xylem, or living cell interiors whose electrical charge separation has been destroyed by stylet action) (Backus *et al*. 2009; Miranda *et al*. 2009; Pearson *et al*. 2014). Interestingly, at the intermediate Ri level of 108 Ohms with the AC-DC monitor, waveform voltage level can be either positive-going only or both negative- and positive-going, depending upon the insect (i.e., probably quality of wiring job) or probe.

Another general characteristic is presence of a landmark waveform termed the X wave (Backus *et al*. 2009). Originally identified by McLean & Kinsey (1967), an X wave is a complex, highly visible, stereotypically repeating waveform that is species-specific and (to date) performed only by salivary sheath feeders (McLean & Kinsey 1967; Backus & Bennett 2009). An X wave marks contact with and penetration of the stylets into a preferred cell type for eventual, sustained ingestion. Accordingly, a true X wave marks the transition between pathway and sustained ingestion phases; both are performed in an acceptable 'target' cell of the preferred ingestion cell type. For all sheath-feeding hemipterans, X waves provide a visual portrayal of the behavioral acceptance process for an ingestion site. Thus, they represent a series of behaviors that: (1) sensorially test and judge the acceptability of a phloem sieve element or xylem vessel element (depending on the ingestion preference of the species), (2) secure a firm attachment to that cell, and (3) begin overcoming any challenges or defenses presented in the cell by the plant (Backus *et al*. 2009). Identification of the sharpshooter X wave marked the first time that such specialized contact and acceptance behaviors have been demonstrated for xylem vessels by preferential xylem-ingesters; all other X waves have been defined for preferential phloem-ingesters (e.g. aphids, deltocephaline leafhoppers).

APPLICATIONS OF EPG TO APHID BEHAVIOR AND IPM

Since the earliest years of electropenetrography, aphids have been widely used as models for studying hemipteran feeding behaviors. The relatively large size and low mobility of aphids makes them perfect for EPG studies. Today, virtually everything known about aphid feeding behavior can be attributed to EPG. Feeding activities of aphids are composed of several sequential behaviors. After an aphid make contacts with a plant, it antennates, using chemosensory hairs to sense gustatory cues (Powell & Hardie 2002). During the first few probes, an aphid will intermittently puncture living plant cells (Tjallingii 1985b), and plant fluids then are taken up to the precibarium (or antecibarium) to make contact with chemosensilla therein; the aphid then will decide to leave the plant or continue probing into the vascular tissues (Powell *et al*. 2006).

The following review summarizes and

synthesizes information about aphid feeding from all three generations of EPG technology. Today, aphid waveforms are classified into three main phases, including: (1) pathway phase, (2) phloem phase, and (3) xylem phase, plus a transition phase (X wave) that alternates between pathway and phloem. Pathway phase comprises waveforms A, B, C, F, and potential drop (pd) (Figs. 3A–3D) (Tjallingii 1978); phloem phase consist waveform E1 and E2 (Figs. 3E–3H); xylem phase consists only of waveform G (Fig. 3J) (Tjallingii 1987). Although the waveforms can be readily categorized by human eyes, it is difficult to define each of the waveform explicitly. In addition to subjective categorization, crude definitions based on relative amplitude, frequency, and voltage levels are used to characterize each waveform (Tjallingii 1978). Almost all aphid species studied show very similar waveforms, representing distinct, corresponding behaviors. The consistency among aphid species is a powerful advantage for selection of aphids as the model for EPG studies, as well as for application to integrated pest management tactics against these important pests.

Aphid waveforms and their biological meanings:

Pathway phase: A, B, and C. In order to locate and establish sustained phloem ingestion, aphids use the salivary sheath strategy of feeding (thus, secreting both sheath and watery saliva), performing intercellular stylet penetration during most of the stylet probe/penetration (individual stylet insertion), simultaneously secreting sheath and watery saliva (Moreno *et al*. 2011), with intracellular penetration more rarely (Fig. 3A) (Pollard 1973). Onset of a stylet probe usually takes place within 1 min after the aphid makes proboscis contact with the plant (Powell *et al*. 2006). Penetration of stylets causes an abrupt, positive-going voltage change. Following initial penetration, waveforms A, B, and C (Figs. 3A and 3B) are usually observed (Tjallingii 1978). These waveforms are always performed in consecutive sequence, and together are referred to as 'pathway activities' of aphid stylet penetration (Tjallingii & Hogen Esch 1993). A, B, and C were highly compressed and combined as a single waveform (called Salivation) in the early years of EPG, due to the slow speed of then-prevalent strip chart recorders; thus it is unknown whether the three waveform types were distinguishable using the low Ri level of the first-generation AC monitor (McLean & Kinsey 1967). Although the detailed activities underlying A, B, and C are still to be unraveled, their importance prior to sustained phloem sap ingestion is certain. Details about the route and positions of the stylets within plant tissues can be found in Tjallingii & Hogen Esch (1993).

Pathway phase: pd. During pathway, especially waveform C (which represents the majority of pathway), there are often numerous intracellular punctures of cells along the stylet route, represented by potential drops (pd) (Figs. 3B and 3D), which last for 5–15 s. The first pd can take place as soon as 10 s after initiating stylet probing, but it usually occurs within 1 min after penetration (Fig. 3B). Parallel lines of evidence over several papers support that these sudden voltage drops are caused by the puncture of parenchyma or epidermal cell membranes. For example, by comparing waveforms of aphids on artificial diets, protoplast suspensions, and plants, Tjallingii (1985b) was able to correlate waveform pd with the presence of living cells. In addition, the voltage level of pd is usually lower than that of waveform C by 100–180 mV; this voltage level resembles the membrane potential of plant cells (-100 to -200 mV), as well as that of waveform E (discussed below), when stylets are located inside a phloem sieve element (Tjallingii 1982). It was thus concluded that pd was caused by the change of potential from intercellular regions into intracellular ones (Tjallingii 1985b). Further support for the hypothesis occurred when numerous mesophyll punctures were observed along the salivary

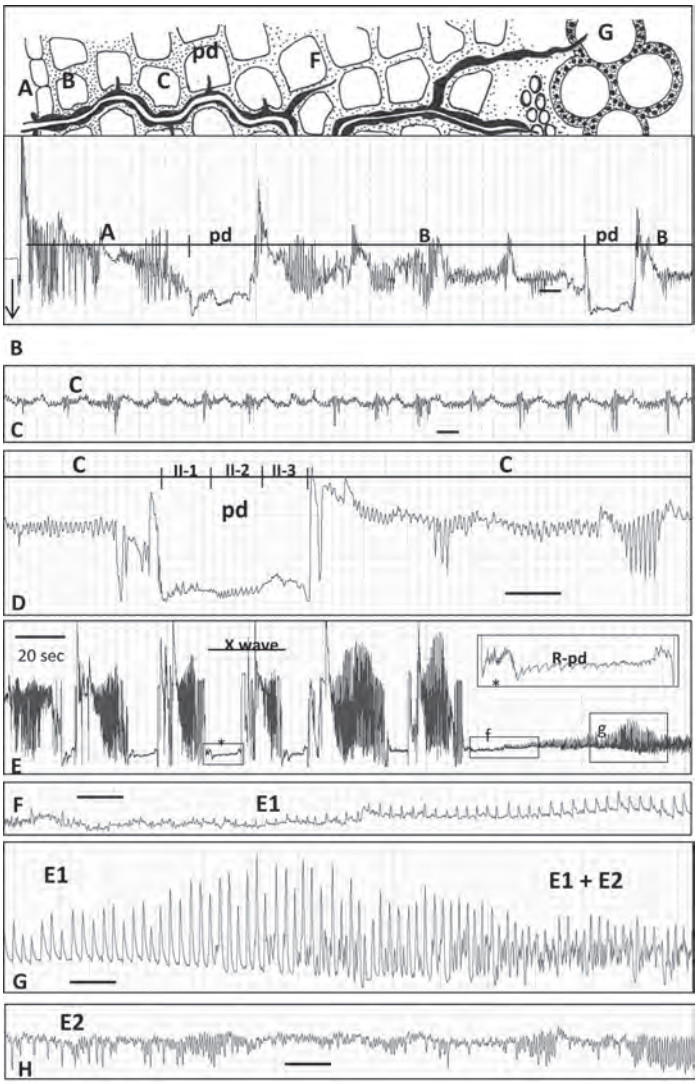

Fig. 3. Summaries of typical EPG waveforms (using an AC-DC monitor at Ri 109 Ohms) from stylet probing of the pea aphid, *Acyrthosiphon pisum*. (A) Drawing of a hypothetical salivary sheath/stylet pathway to xylem and phloem cells with labeled portions of the sheath to represent typical locations of the waveforms (letters) recorded during each stage of probing. The sheath is hollow because the stylets were removed during E2, to identify the final sheath branch into a phloem sieve element cell. Filled, abandoned, branches represent stylet derailment during probing (waveform F; not pictured below) and xylem ingestion (waveform G, not pictured below). All scale bars represent 2 s of recording, unless otherwise stated. (B) Start of a stylet probe (voltage increase after flat baseline/non-probing; baseline denoted by an arrow). Note that waveform A is quite brief, and that the first intracellular puncture (pd) occurred within the first 30 s. The two pd's are also noticeably below the baseline level (therefore, negative-going). (C) Waveform C, much later in pathway; note that its amplitude (Y axis) is shorter than that of either A or B. Amplification of parts b and c is 4×. (D) Short section of waveform C with embedded pd (with pd sub-phases), at twice the amplification (8×) of part c. (E) Compressed view of 5.5 X waves at the end of pathway in the same probe, prior to E1 then E2. Boxed area of waveform with * is enlarged in the inset box, showing details of sub-phase II of the R-pd. Lettered boxes on waveforms are expanded in the next sections. (F) Start of E1, with positive-going peaks, showing the evolution of its appearance over time. Amplification of sections f–h is 8× (double that of section e). (G) Tallest peaks of E1 in transition to E2, during which the E1 and E2 peaks overlap and merge. (H) E2 alone, much later in the probe. An appearance similar to classical E2 (using the DC monitor), with negative-going peaks, occurs at the beginning of this excerpt near the letter h; other variations in appearance are typical of pea aphid recordings with the AC-DC monitor, probably due to greater sensitivity and rapid response time of modern amplifier electronics.

sheath (i.e., route of stylets) using electronic microscopy (Tjallingii & Hogen Esch 1993).

Potential drops are subdivided into three sub-phases (or sub-types), namely I, II, and III (Fig. 3D). Sub-phase I is the abrupt, descending drop of the waveform; sub-phase II is the short period of stable waveform at a lower potential level; sub-phase III is the sudden rise/recovery of potential back to the original (extracellular) level. The activities reflected by pd sub-phases I and III are the penetration of stylets through the plasmalemma and the withdrawal of stylets, respectively (Tjallingii 1985b). Sub-phase II is a more complicated waveform consisting of three distinctive parts, II-1, II-2, or II-3 (Fig. 3D) (Powell *et al*. 1995). Because it is very difficult to correlate stylet activity in a cell within 5–15 s by conventional methods (e.g. stylectomy), different approaches using aphid-transmitted non-persistent viruses as phloem markers have been used to elegantly correlate the behavior of aphids during sections of pd sub-phase II (Martin *et al*. 1997). Emulating the idea from earlier, AC monitor research of Scheller & Shukle (1986) (discussed below), aphid-virus combinations were used from two pathosystems, *Aphis gossypii* Glover transmitting *Cucumber mosaic virus* (CMV) and *Myzus persicae* Sulzer transmitting *Potato virus Y* (PVY). In the first study, non-viruliferous aphids were recorded during an acquisition access period on virus-infected plants, which was terminated at the end of sub-phase II-1, II-2, or II-3 in the first-recorded pd, and the aphid was later used to test inoculation efficiency. In a second experiment (similar to the acquisition experiment), viruliferous aphids were recorded during an inoculation access period, which was terminated at the end of sub-phase II-1, II-2, or II-3 in the first pd. The result indicated that pd subphase II-3 was correlated strongly with acquisition thus indicating intracellular ingestion, and pd subphase II-1 corresponded to viral inoculation thus indicating intracellular salivation. These findings suggest that the behaviors during sub-phase II-1, II-2, or II-3 are likely salivation, unknown behavior, and ingestion of plant cell contents, respectively (Martin *et al*. 1997). Harris later hypothesized that sub-phase II-2 represents egestion of fluid from the precibarium in the functional foregut (Harris & Harris 2001). This hypothesis has not been experimentally tested in aphids, however, the existence of egestion in hemipterans has recently been proven (Backus *et al*. 2015).

The pd waveform is best viewed using high Ri levels, such as with the DC (10^9 Ohms) or AC-DC monitors (10^8 Ohms or higher), because all three sub-phases are visible. However, sub-phase II-2 was also detectable using the AC monitor (Reese *et al*. 2000) at lower Ri level (10^6 Ohms). Sub-phases I and III (the fall and rise) were not visible, but a version of sub-phase II (a mostly-flat line, without divisions into the three sections) was visible. When pd's are recorded at multiple Ri levels using the third-generation AC-DC monitor, the depth of the pd and amount of detail of sub-phase II sections increase as Ri is stepped up from 10^6 to 10^{13} Ohms (Backus & Bennett 2009). This confirms that the sub-phase II details are emf components.

Pathway phase: F. Waveform F is occasionally observed during pathway activities, and it was never recorded in feeding of artificial diet. The duration of waveform F varies from minutes to hours and does not lead to phloem penetration. All the aphid species tested in the study of Tjallingii (1987) perform this waveform, but showed different frequencies among host plants. According to histological studies, the aphid's stylets were always located within the intercellular space of epidermal cells with thick cell walls and obscure middle lamellae. However, results from radioisotope intake experiment shows a ten times higher rate of plant sap uptake during F than during waveform A, B, and C. Based on limited information, waveform F is presumed to be the interaction between stylet and particular cell walls while the stylets are struggling to

free themselves from a tight space (Tjallingii 1987). The waveform is now termed 'derailed stylet mechanics'. Waveform F was termed 'non-phloem ingestion' in studies with the AC monitor, and was not distinguishable from waveform G (see below) using the lower Ri level of that early monitor.

X wave, an inter-phase transition. The first identification of an X wave as a landmark waveform was made with the AC monitor at low Ri level. Although the behavior represented by the X wave was unknown, it was found to precede ingestion from phloem sieve elements by *Acyrthosiphon pisum* Harris, 100% of the time (McLean & Kinsey 1964, 1967). A study of feeding by *Sitobion avenae* F. (Scheller & Shukle 1986) elegantly correlated X waves with inoculation by using the aphid-transmitted plant virus, *Barley yellow dwarf virus* (BYDV) as a marker. BYDV is persistent-circulatively transmitted by *S. avenae* (among many aphids), and is phloem-limited. Virions are acquired by ingestion of phloem sap, and inoculated (after a latent period for virus circulation) via salivation into phloem sieve elements (Sylvester 1980). EPG waveforms were recorded for 90 min while viruliferous aphids were feeding on healthy test plants. Results showed that X waves were prerequisite for inoculation, thus they represented both contact with and salivation into phloem sieve elements. A single X wave resulted in a 65% chance of inoculation and subsequent infection, regardless of the duration of the X wave. The probability of inoculation/infection increased with increasing numbers of X waves (Scheller & Shukle 1986).

A deeper understanding of the relationship between X waves and pd's began to develop when Reese *et al*. (2000) identified the first part of the X wave as a pd that was significantly longer than earlier pd's in the same probe. The second part of the X wave was a return to C with a longer, more elaborate comb-like portion than in pre-X pathway C. Thus, the long-duration components of the X wave made the waveform easily identifiable, even in the highly compressed recordings of the AC monitor. Identification of an unusual form of pd, the R-pd, finally explained the mechanism of the aphid X wave. R-pd's have been observed in *A. pisum, Brevicoryne brassicae* (L.), *Drepanosiphum platanoidis* (Schrank), and *Periphyllus acericola* (Walker) (Tjallingii & Gabryś 1999; Backus & Bennett 2009). This unusual pd is an extended form of normal pd, with longer subphase II and smaller amplitude (Fig. 3E, inset box with *). R-pd is always part of an X wave (Fig. 3E), thus always precedes waveform E1, and occurs more often on mature and old leaves (less preferred feeding sites) than young leaves and stems by *B. brassicae*. Together with the results from electron micrographs, Tjallingii & Gabryś (1999) concluded that the R-pd waveforms (and thus X waves) were caused by repeated punctures into and out of the same sieve element. Accordingly, the aphid X wave represents a transition alternating between pathway and phloem phases. Although the function of the X wave behavior is not yet proven, it is likely to be part of the cell-acceptance process of aphids, as with X waves of other sheath-feeders (Backus *et al*. 2009).

Phloem phase: E1 and E2. Early studies using the DC monitor showed that phloem penetration was associated with waveform D + E (pd) (Kimmins & Tjallingii 1985), which was later renamed and divided into waveforms E1 and E2 (Tjallingii 1990). Usually, E1 begins right after the last X wave; after a certain period of E1, E2 will ensue on a suitable plant. E1 and E2 are composed of very regular, repetitive peaks (Figs. 3E–3H). The amplitudes of waveforms E1 and E2 are low compared to waveforms A, B, and C. The main differences between the two waveforms are their patterns and electrical origins. Waveform E1 is an emf-dominated waveform, whereas E2 is an R-dominated waveform, which means waveform E2 changes according to the adjustment of substrate voltage, and waveform E1 is less affected (Tjallingii 1990). Waveform E2

always occurs after E1, and never the reverse. Although waveforms E1 and E2 seem to be highly associated, performance of waveform E1 does not necessarily lead to E2 (Prado & Tjallingii 1994). Early studies with the AC monitor could not distinguish between E1 and E2, due to low Ri level; the combined waveform was referred to as 'phloem ingestion' due to histological correlation with phloem; however this waveform is now better described as phloem phase.

The biological meanings of waveform E1 and E2 are well understood. They were first associated with sap ingestion and salivation, respectively, based on experiment result using radioactive labeled feeding solutions and electromyograms (EMG) of salivary pump muscle (Tjallingii 1978).In the early DC literature, duration of waveform D (today equivalent to E1 and E2, together) was positively correlated with the amount of radioactive orthophosphate inside aphids, and the frequency of the fluctuation pattern coincided with pattern of salivary pump movement in the EMG. The positions of the stylets were demonstrated by stylet amputation and electron microscopy observation. When aphid stylets were amputated 7 min 30 s after the start of waveform E1 and E2, phloem sap exuded from the cut end of the stylets due to the phloem turgor pressure. Electron micrographs clearly showed that the stylets, during waveform E1/E2, were inside the lumen of sieve element (Kimmins & Tjallingii 1985) and no sheath saliva was found inside sieve element (Tjallingii & Hogen Esch 1993).

In the study of Prado & Tjallingii (1994), they reported that the behaviors during waveform E1 and E2 were correlated with salivation (E1) and ingestion simultaneous with watery salivation (E2) in phloem sieve elements, after repeating and expanding upon the original study of Scheller & Shukle (1986). Again, they used the aphid-transmitted plant virus, *Barley yellow dwarf virus* (BYDV), but used the bird cherry-oat aphid, *Rhopalosiphum padi* (L.), as the vector. This time, either acquisition or inoculation behaviors were terminated by pulling the aphid away from the plant at a specific time point. Results showed that when waveform E2 lasted for 10 min or longer, the acquisition efficiency was 58% and had the strongest relation to virus acquisition, whereas the duration and numbers of waveform E1 showed relevance for virus inoculation (Prado & Tjallingii 1994), similar to X waves in Scheller & Shukle (1986). When combined, the results of Scheller & Shukle (1986) and Prado & Tjallingii (1994) demonstrate that repetitive sieve element punctures (i.e., R-pd's in X waves) and continuous watery salivation (i.e., waveform E1) both represent the complicated behavior prior to acceptance of a sieve element cell, as described above under 'X waves.'

In a study using *Aphis fabae* Scopoli, the average time for aphids to access phloem was 45 min per probe, but the average time to achieve sustained phloem ingestion usually took 3–4 h (Tjallingii 1994). An intriguing relationship was found between waveform E1 and host plant acceptance. Aphids that feed on host plants with varying degrees of resistance usually perform longer durations of E1 events, and sometimes shorter or absent events of E2 (Nielson & Don 1974; Niassy *et al*. 1987; Ryan *et al*. 1987; Dixon *et al*. 1990). This indicates the importance (and potential mechanisms) of salivation against host plant defenses responses and any resistance factors located in phloem sap.

Xylem phase: G. Waveform G is produced occasionally by aphids on diets and plants, and is presumed to be xylem ingestion by histological evidence, which correlated the waveform and the location of stylets in xylem. In addition, starving the aphid or putting aphids in a desiccated environment before the experiment can significantly increase the numbers and durations of waveform G events (Spiller *et al*. 1990). The differences between waveform G and E2 are as follows: (1) more sinusoidal shape, (2) higher amplitude, (3) higher potential level (waveform G has an

extracellular potential level), and (4) more irregular pattern (Tjallingii 1987). As major phloem-ingesters, most of the aphids used in EPG studies didn't show much xylem ingesting. However, alate (winged) *A. fabae* tend to ingest from xylem after they settle on plants (about 60% of individuals), whereas no apterous individuals performed waveform G (Powell & Hardie 2002). The authors concluded that there could be an evolutionary benefit of xylem ingestion for aphid dispersal by different morphological groups of aphids.

APPLICATIONS OF APHID EPG TO IPM RESEARCH

In addition to basic feeding behavior research, the behavioral characteristics of aphids uncovered by EPG can be applied across a wide discipline of practical studies. One of the major applications of EPG is in evaluation of host plant resistance. Aphid's behavioral characteristics on aphid-susceptible and -resistant cultivars or host and non-host plants provides the researcher a time-saving tool to evaluate the degree of resistance (Nielson & Don 1974; Lohar & Kawada 1987; Peters *et al*. 1988; Montllor & Tjallingii 1989; Annan *et al*. 2000; Wang *et al*. 2004), or to identify the location of resistance (van Helden & Tjallingii 1993; Caillaud *et al*. 1995; Kaloshian *et al*. 2000; Zehnder 2001; Sandanayaka *et al*. 2003; Le Roux *et al*. 2008; Lightle *et al*. 2012; Koch *et al*. 2015). Another agricultural application of EPG is evaluation of feeding behavioral changes by the application of pesticides or repellents (Holbrook 1977; Reuter *et al*. 1993; Harrewijn & Kayser 1997; Abraham & Epperlein 1999; Kang *et al*. 2012). Aphids are major vectors of many plant pathogenic viruses that cause enormous loss worldwide. The viral transmission behaviors of aphids have been extensively studied via EPG. Aphid behaviors that are correlated with viral acquisition or inoculation can be identified (Hodges & McLean 1969; Holbrook 1978; Powell 1991; Prado & Tjallingii 1994; Martin *et al*. 1997), and differences in feeding behavior of viruliferous/non-viruliferous aphids or on virus-infected/virus-free host plants can be disclosed (Ullman *et al*. 1988; Blua & Perring 1992; Quiroz *et al*. 1992; Boquel *et al*. 2011, 2012; Hu *et al*. 2013).

EPG can also be applied to studies of ecology and evolutionary biology. The results of interactions between aphids and plants can often be quickly inferred by EPG, which has been mentioned in previous paragraph. In addition, intraspecific interactions or interspecific interactions of aphids were studied by comparing behaviors recorded using EPG monitors (Chongrattanameteekul *et al*. 1991; Formusoh *et al*. 1992; Prado & Tjallingii 1997; Morris & Foster 2008; Brunissen *et al*. 2009). Moreover, behavioral variations were used to reflect genetic variations and to test hypothesis of host specialization, diversification, and sympatric/allopatric speciation of aphids (Caillaud 1999; Powell & Hardie 2000; Funk & Bernays 2001; Tosh *et al*. 2001, 2003; Halarewicz & Gabryś 2012; Kordan *et al*. 2012).

PROSPECTS FOR APHID EPG RESEARCH IN TAIWAN

Although EPG has been used for aphid studies for over 50 years, until recently, there was no research in Taiwan that involved EPG. Aphids are among the most important pests in Taiwan. They can cause severe yield loss by direct feeding damage or transmitting plant viruses (Lee 1986; Bau *et al*. 2004; Deng 2011). There are seven aphid-transmitted plant viruses of economic importance in Taiwan, i.e., *Cucumber mosaic virus*, *Papaya ringspot virus* W strain, *Papaya ringspot virus*, *Potato virus* Y, *Turnip mosaic virus*, *Zucchini yellow mosaic virus*, and *Banana bunchy-top virus* (Deng 2011; Tsai & Chen 2011). Researchers have been struggling for years to find ways to control these viral diseases. One of the most effective and fundamental ways of dealing with viral diseases is breeding resistant strains of

crop plants (Niassy *et al*. 1987; Li *et al*. 2007; Smith & Boyko 2007). Crops resistant to either insect vector or virus can benefit agricultural production by decreasing the transmission of virus; those resistance traits are sometime mutually inclusive (Chen *et al*. 1996). EPG can provide a rapid preliminary evaluation of the crops for aphid resistance in most of the aphid-plant combinations, due to the similarity of waveforms among aphids. Most of the aphid-transmitted viral diseases in Taiwan are non-persistently transmitted, which means the acquisition or inoculation can take place early in a single probe, most likely during pd's in pathway phase. In this case, future EPG research would need to focus on resistance factors located in the epidermis or on the surface of plant that can prevent the transmission behavior.

EPG can also be valuable for study of residual pesticide effects, enabling researchers to evaluate the potential of pesticides for viral disease control. Despite the fact that some of the major aphid vectors in Taiwan have already been studied, the effects of viruses on behaviors of aphids have not been quantified using EPG. EPG is a promising method for researcher to decipher the interaction between aphid and specific virus, and might lead to influential discoveries in basic biology and beneficial application in agriculture.

The above-mentioned examples clearly show the potential and importance of EPG in understanding the behaviors of aphid pests in Taiwan, and it is certain that valuable information will continuously be discovered by the use of EPG.

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昆蟲刺探電位圖譜 (EPG)：刺吸式害蟲取食行為鑑定新技術 †

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摘要

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由於無法直接觀察半翅目昆蟲特化口針在植物組織的刺探與穿刺過程，致使研究此類昆蟲的取食行為、對植物損害以及傳播植物病原的相關研究，存在極大挑戰，及至 50 年前所發明昆蟲刺探電位圖譜 (electrical penetration graph; EPG) 監測儀，才克服這些研究上的障礙。如今 EPG 主要以 3 個方向發展半翅目害蟲整合管理 (IPM) 新策略，包括 (1) 對特定半翅目昆蟲之取食行為與造成植物傷害機制未明時，EPG 是可有效解決的工具；(2) 解決上述問題之後，EPG 可被應用於分析殺蟲劑或拒食劑對害蟲行為所造成的影響，並進一步評估其他化合物對於特定取食行為造成危害與傳病的原因；(3) EPG 能被應用於不同植物品系抗蟲程度的評估，包括基因改造表現生物殺蟲成分的作物。本回顧報告主要目的包括 (1) EPG 的原理以及歷史，特別針對第 3 代 AC-DC 儀器進行介紹；(2) 以蚜蟲為模式系統，作為瞭解那些波形可自 EPG 獲得；(3) EPG 可應用在台灣農業的那些需求，特別是對於那些具有經濟重要性的蚜蟲。本文係首次提出 3 型 EPG 在蚜蟲研究概況的回顧研究報告。

關鍵詞：昆蟲刺探電位圖譜、半翅目、取食行為。

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