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Linear-motion tattoo machine and prefabricated needle sets for the delivery of plant viruses by vascular puncture inoculation

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Abstract Vascular puncture inoculation (VPI) of plant viruses previously has been conducted either manually or by use of a commercial engraving tool and laboratory-fabricated needle arrays. In an effort to improve this technique, a linear-motion tattoo machine driving industry-standard needle arrays was tested as a means of delivering plant viruses into maize and small grain seed embryos. The new method was applied in the successful transmission of maize rayado fino virus (MRFV), the type member of the genus *Marafivirus*, from an archived sample to maize. Subsequent transfer of MRFV from the sap of an infected plant using the method produced an average infection rate in maize of 70% (range 39–93%). Maize, oat, and triticale were successfully infected with oat blue dwarf virus (OBDV) using the method; similar infection rates were observed between maize seeds inoculated with the tattoo machine and those inoculated with the engraving machine when using prefabricated needle arrays. No infection was obtained in repeated tests with barley yellow dwarf virus (BYDV-PAV) or cereal yellow dwarf virus (CYDV-RPV) using either sap or RNA from infectious cloned cDNA. Replacement of the original engraving-tool with a linear-motion tattoo machine in VPI provides greater flexibility and convenience in a quiet, readily-available instrument, while improving reproducibility through the use of prefabricated needle arrays.

Keywords Luteovirus · Marafivirus · Mechanical inoculation

Abbreviations

BYDV	barley yellow dwarf virus
CYDV	cereal yellow dwarf virus
MRFV	maize rayado fino virus
OBDV	oat blue dwarf virus
VPI	vascular puncture inoculation

Introduction

The natural spread of plant viruses between host plants is enabled by numerous vector organisms, as well as by mechanical wounding (Agrios 2005). Transmission by rub inoculation on the leaves of host plants has facilitated experimental research on plant viruses for decades. Yet many plant viruses, typically among those transmitted by insects, cannot be propagated in this manner (Matthews 1991).

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Improving on the "embryo wounding" technique for the infection of maize with white line mosaic virus (Zhang et al. 1991), Louie (1995) demonstrated that fine insect pins of 0.15 to 0.35 mm, when affixed in an array to an oscillating engraving tool, were capable of delivering a range of viruses into maize seeds resulting in the infection of plants arising from these seeds. Many of the viruses transmitted in that study cannot be transmitted by rub inoculation; for these viruses, therefore, the technique experimentally circumvented the need for the natural insect vector in virus transmission. Subsequent reports confirmed the efficacy of the "vascular puncture inoculation" (VPI) technique, which has been used to initiate plant virus infections from pure and crude preparations of plant viruses (Madriz-Ordeñana et al. 2000; Redinbaugh et al. 2001; Louie et al. 2006) as well as from natural and synthetic viral DNA and RNA (Redinbaugh et al. 2001; Redinbaugh 2003). Drawbacks of VPI as it has been described to date are the excessive noise associated with the recommended engraving tool and the need to fabricate needle arrays, which are fragile, may exhibit considerable between-array variability, and are time-consuming to produce.

Recently, we reported on the first infectious cDNA clone of a marafivirus, oat blue dwarf virus (OBDV; Edwards and Weiland 2010). We were interested in employing VPI for the delivery of OBDV and other marafiviruses in our laboratory, but desired a uniform, reproducible format that could be used to test large numbers of mutant virus genomes for genetic analysis. An investigation of devices with capabilities similar to those of an engraving tool indicated that a tattoo machine might provide an effective substitute for VPI of plant viruses. Indeed, tattoo machines have been employed in animal virology for decades in the delivery of viruses and viral antigens for the induction of infection and immune responses (Kidd 1938; Quaak et al. 2009; Pokorná et al. 2009). By coupling linear-motion tattoo machines with standard prefabricated tattoo needle arrays, it was conceivable that VPI of plant viruses could be achieved.

Using a linear-stroke tattoo machine driving industry standard 4- and 7-needle arrays, we demonstrate successful VPI in the transmission of the marafiviruses OBDV and maize rayado fino virus (MRFV) to maize. We further demonstrate transmission of OBDV to seeds of oat and triticale that results in plant infections exhibiting characteristic symptoms of blue dwarf disease. As the tattoo industry is well-developed globally, it is anticipated that the availability of "turnkey" machines and accessories for VPI will enable plant virology laboratories to more readily integrate the technique into their research programs where desired.

Materials and methods

Minuten pins (0.15 or 0.2 mm; BioQuip Products, Rancho Dominguez, CA) were arranged in a 4-needle linear array and affixed to a nylon rod with two-component epoxy and the dried assembly inserted into an engraving tool (Ideal Industries, Sycamore, IL) for use in standard VPI. Alternatively, the bar of a commercial tattoo needle array was cut to permit insertion into the engraving tool mechanism. For tattoo machine-assisted VPI, a linear-motion tattoo machine providing a 2.5 mm stroke length (NeoTat^{TM} machine, TapTat Daddio Inc., Miami, AZ USA) with an accompanying variable voltage power supply, interfacing power cable, and control footswitch for hands-free on/off operation of the machine was used. Most commercial tattoo machines are designed to accept industry standard tubes and needle arrays for tattoo artistry. Prefabricated needle arrays used in the present study were either "7-needle weaved magnum shader" arrays (4 needles above and 3 below in an offset pattern; Fig. 1) or "4-needle flat shader" arrays constructed with # 8 bugpins (0.25 mm needle diameter, medium taper) inserted into a flat tip plastic tube with an integrated grip. Tubes and prefabricated needles are disposable and available in pre-sterilized blister packs (WorldWide Tattoo, Inc., City of Industry, CA). The 7-needle array was used for all maize seed inoculations, whereas the 4-needle array was used for small grains. Depending on the experiment, voltage delivered to the machine was adjusted to provide oscillation of the needle array at a rate of 2,400 cycles per minute (cpm) or 7,200 cpm as determined with a stroboscope.

Barley stripe mosaic virus (BSMV), wheat streak mosaic virus (WSMV), and OBDV, all maintained in culture in our laboratory, a US isolate of MRFV (Edwards and Weiland 2011), and BYDV-PAV and CYDV-RPV (both kindly provided by J. Anderson, USDA-ARS/Purdue Univ.) served as inoculum sources. BYDV and CYDV were provided both in symptomatic leaves and as cloned DNA (plasmids pPAV6 and

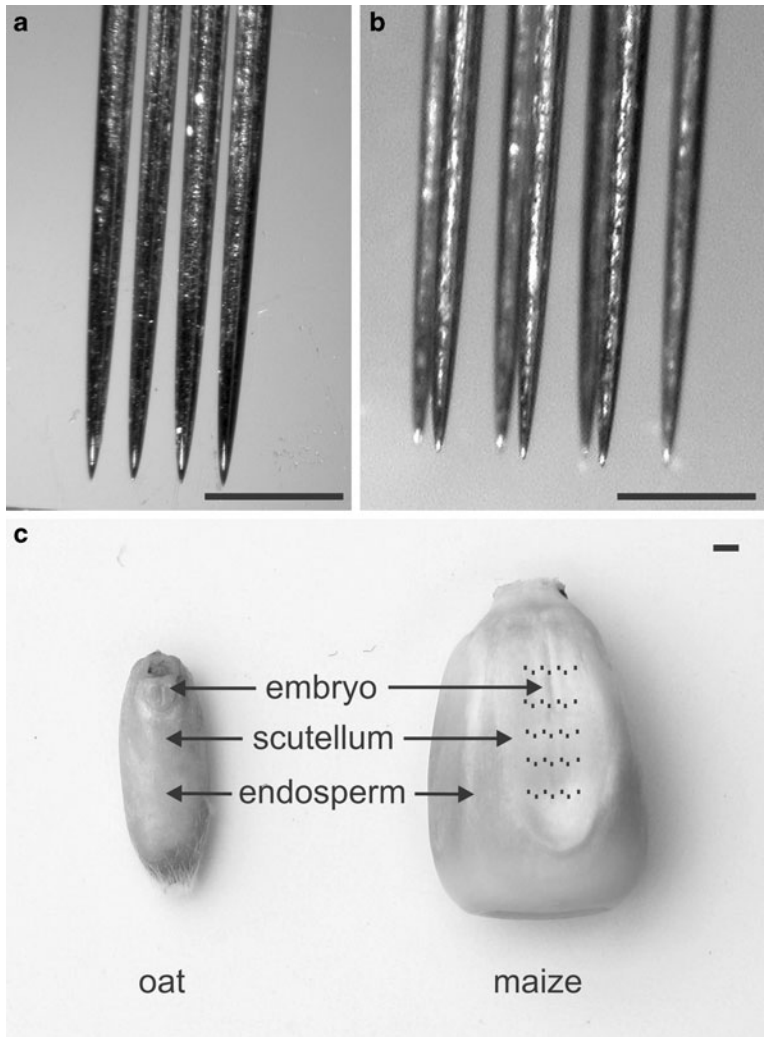

Fig. 1 Relative size and configuration of prefabricated needles (4-needle flat shader, panel **a**; 7-needle weaved shader, panel **b**) and target seed tissues (panel **c**) in the development of tattoo machine-assisted vascular puncture inoculation. The vascular tissues embedded within the scutellum and the embryo are the targets for delivery of inoculum. The 7-needle weaved shader has 4 needles offset from 3 needles in a stacked configuration and an approximation of the puncture pattern is shown by the dots on the maize seed image. Bars=1 mm

pRPVNY83) from which infectious RNA can be transcribed. Transcript RNA used in this study was synthesized as described (Weiland and Edwards 1994) except that cap analog was omitted from BYDV and CYDV transcriptions. An MRFV-symptomatic plant validated by ELISA was harvested and the leaves were freeze-dried in order to serve as an inoculum stock. Inoculum was either an extract prepared from an infected plant in 10 mM potassium phosphate buffer as described by Louie (1995), concentrated virus prepared by crude virus precipitation from extract using polyethylene glycol (PEG; Edwards and Weiland 2010), or transcript RNA (adjusted to 1 μg/μl in ddH_2O) derived from clone pOBDV-2r (Edwards and Weiland 2010). Seed of maize (*Zea mays* hybrid 'Silver Queen'), hulless oat (*Avena sativa* cv 'Paul') and triticale (×*Triticosecale* Wittmack) were incubated in distilled water containing 1X strength Fungigone™ (Bio-World, Dublin OH, USA) at 30 C for 2 h. Prior to inoculation, seed was tamped dry and arranged on a glass plate with the embryo-side up as indicated by Louie (1995).

After depositing inoculum (average of approximately 2 μl per seed) on the surface of the embryo, the machine was turned on and the oscillating needles pushed directly through the inoculum at 5 different sites along and through the embryo (3 sites for small grain seeds) and about 1 mm below the surface of the embryo (Fig 1; Louie 1995). The needle and grip assembly was held at a ~45° angle with respect to the seed during inoculation, while a forceps held in the

opposite hand was used to immobilize the seed. Inoculated seeds subsequently were arranged on water-saturated discs of germination paper in 15 cm dia. glass Petri dishes (30 per plate for maize and 50 per plate for small grains) with the embryo side up. Covered dishes containing seeds were placed into a humidity chamber and incubated at 30°C for 24 h, during which time most seeds germinated. All seeds were subsequently sown in a commercial soil mix in a greenhouse or growth chamber at 24°C and 14 h day length. Plants were analyzed visually and with double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) at 12–15 days post-inoculation (dpi) (Clark and Adams 1977); samples with absorbance values 3-fold or greater above those of uninfected control samples were considered virus positive. MRFV induced typical striped stippling in infected maize and OBDV-infected oat and triticale plants exhibited characteristic dwarfing and vein thickening. OBDV is only infrequently and faintly symptomatic in maize (Edwards and Weiland 2009).

Results and discussion

Initial attempts at VPI using an engraving tool and laboratory-fabricated needles as described by Louie (1995) for transmission of OBDV were successful, but resulted in inadequate infection rates for our intended application. Additionally, although the VPI technique has been used successfully for transmission of several viruses (Louie 1995; Madriz-Ordeñana et al. 2000; Redinbaugh et al. 2001; Redinbaugh 2003; Seifers et al. 2004; Louie et al. 2006), the engraving tool is noisy and somewhat cumbersome to use. Moreover, laboratory fabrication of needle arrays can be time-consuming and a source of experimental variability. Commercial motor-driven tattoo machines, on the other hand, are quiet and easy to use and prefabricated needle arrays in numerous configurations are readily available.

In order to validate the use of a tattoo machine for VPI, we attempted to transmit viruses not otherwise mechanically transmissible. Our typical configuration employed a linear motion tatoo machine, a variable voltage power supply, and an on/off control footswitch. As no standard oscillation rate for artistic use exists within the tattoo industry, we initially set the voltage at the midpoint between zero and the maximum voltage provided by the power supply (18 V). Subsequent examination of the oscillating needles with a stroboscope indicated a speed of 2,400 cycles per minute (cpm) at this setting. Using the 7-needle array, freshly prepared extract from a MRFV-infected maize plant was delivered to 120 maize seeds in three separate experiments (40 seeds per experiment). Typically a range of 30–95% of the seed survived the procedure to produce seedlings. Since the germination rate of the healthy seed was 95–98%, it is likely that the reduced germination rate of seeds inoculated with both the engraving tool and the tattoo machine was due to mechanical damage to the embryo. A proportion of the plants generated from treated seeds exhibited typical MRFV symptoms, a result confirmed by ELISA. The rate of infection for each of the three experiments relative to the number of surviving plants was 39% (9/23), 80% (28/35) and 93% (28/30), indicating successful use of the tattoo machine for VPI. These transmission rates compare very favourably with the average 43.2% transmission of MRFV to maize obtained by Madriz-Ordeñana et al. (2000) and the 1–25% transmission obtained by Louie (1995).

We then investigated the usefulness of the tattoo machine and the 7-needle array for infection of maize with OBDV, as we previously had documented the ability to achieve this using an engraving tool (Edwards and Weiland 2010). Inoculum was either sap from oat leaves infected with OBDV, virus concentrated from the sap using PEG, or infectious RNA transcribed from clone pOBDV-2r. The results are summarized in Table 1. Whereas use of sap prepared from infected leaves generally resulted in the lowest infection rates (range 11–13%), use of concentrated OBDV and transcript RNA (1 μg/μl concentration) resulted in similar rates of infection (30–41% and 28–41%, respectively). Low percent transmission was still detected with an RNA concentration of 0.01 μg/μl (Table 1). These results suggest the feasibility of evaluating transcripts of wild type and mutant OBDV genomes in maize, while permitting leafhopper transmission from maize to common hosts, such as barley and oat (Edwards and Weiland 2009; Edwards and Weiland 2010).

Because evaluation in the absence of leafhoppers may be desirable, additional tests were performed on oat, barley, and triticale with transcript RNA and a 4-needle array. In three independent trials, the rates of infection generated by tattoo-machine assisted VPI

Table 1 Percent infection of plant hosts by oat blue dwarf virus following tattoo-machine assisted vascular puncture inoculation

	Inoculum[a]						
	OBDV sap	OBDV conc	OBD trRNA 0.01	OBD trRNA 0.1	OBD trRNA 1.0		
Host:	maize	maize	maize	maize	maize	oat	triticale
Exp. 1	13% 6/46(70)[b]	41% 11/27(100)	3.4% 2/59(60)	8.6% 4/58(60)	33% 12/36(60)	2% 2/95(100)	6% 6/94(100)
Exp. 2	13% 9/70(100)	32% 19/59(100)	0% 0/43(60)	6.3% 3/48(60)	40.5% 17/42(60)	7% 6/86(100)	18% 16/88(100)
Exp. 3	11% 5/46(100)	29.6% 16/54(100)	1.8% 1/53(60)	5.8% 3/52(60)	28% 15/53(90)	10% 9/92(100)	10% 9/93(100)

[a] Inoculum consisted of sap from an infected plant (OBDV sap), virions partially purified and concentrated from an infected plant (OBDV conc), or RNA transcribed from a full-length OBDV clone (OBD trRNA) and applied at three different concentrations, 1 μg/μl, 0.1 μg/μl, or 0.01 μg/μl

[b] Percent infection with OBDV and number of infected plants/number of germinated seeds with the total number of inoculated seeds in parentheses

ranged from 2% to 10% for oat and from 6% to 18% for triticale (Table 1). No infection was obtained in barley in these tests. While infection rates were not as high as in maize, this represents the first report of direct delivery of OBDV to a small grain species and of the infection of triticale by OBDV.

Based on this success, we investigated application of this method to the transmission of additional viruses. As expected, infection of maize with BSMV and WSMV (both transmissible by rub-inoculation to leaves) was readily achieved using sap prepared from infected plants as inoculum (greater than 50%; not shown). Because of the significant economic impact of BYDV and CYDV in small grain crops, and because no efficient alternative to aphid transmission exists for these viruses, we attempted transmission of these viruses to maize and oat using this method. Numerous attempts were made to infect maize and oat using either RNA transcripts derived from infectious cDNA clones or sap prepared from oat plants infected with these viruses. In 4 replicated experiments (100 seeds treated per inoculum source per experiment), no symptomatic or ELISA-positive plants indicative of BYDV or CYDV infection were observed. It is unclear as to why VPI of BYDV or CYDV should be problematic, although previous attempts at VPI with these viruses also have been unsuccessful (J. Anderson, personal communication). It is worth noting that small accumulations of BYDV may be found in immature phloem (Gill and Chong 1975), but direct necrosis of phloem tissues occurs following what initially appears to be normal differentiation and development of those tissues (Esau 1957). In contrast, OBDV appears to replicate well in immature phloem and induces hyperplasia prior to development of any necrosis within the phloem (Zeyen and Banttari 1972). Differences in pathological effects on phloem tissues infected with yellows viruses versus OBDV may reflect an as yet unknown fundamental difference in their biology that results in the inability to transmit BYDV/CYDV by VPI.

Final tests were conducted to determine if infection rates obtained with the tattoo machine were comparable to those obtained with the engraving tool while using the 7-needle array in both devices, and if these rates were affected by the speed of the tattoo machine. In two independent inoculations of maize seeds with OBDV transcripts, similar infection rates were observed between seeds inoculated with the tattoo machine and those inoculated with the engraving machine (not shown). In the test of oscillation frequency, however, two separate experiments were performed demonstrating a consistent increase in infection rate (from 6 to 18% in one experiment and from 7 to 12% in a second experiment, 100 seeds per experiment) when 2400 cpm was compared to 7200 cpm. For this reason, we have adopted the faster rate of 7200 cpm, which is accompanied by a minimal increase in machine vibration and noise, for our current studies.

This report illustrates the use of a new device and accessories to facilitate VPI of plant viruses to monocots. Replacement of the original engraving tool with a linear-motion tattoo machine provides flexibility and convenience in a quiet, readily-available instrument. The availability of tattoo machines with differing

stroke lengths and a wide variety of prefabricated needle configurations should facilitate optimization for numerous virus-host systems. Although VPI has been successfully used to transmit numerous monocot-infecting viruses, the use of VPI to infect dicots has not, to our knowledge, been reported. It is anticipated that the ready availability of tattoo machines and prefabricated needle arrays will enable the introduction of this technology into laboratories for the study of the many known and emerging plant viruses and virus-like agents of hosts in both plant classes.

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