Evaluation of Ethylene Production in Tobacco and *Arabidopsis* Induced by Particle Bombardment

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Introduction

Particle bombardment is a helpful tool for gene expression studies in plants. It has been successfully used for transforming barley tissues (Skadsen et al. 2002) and characterizing promoter elements that regulate abscisic acid-induced gene expression in barley aleurone (Casaretto and Ho 2003). Researchers have demonstrated that real-time RT-PCR is an effective method for evaluation of mRNA transcript levels in tobacco seedlings after transformation by particle bombardment (Miyamoto et al. 2000). Using this method, they reported that the greatest transcript abundance of the GUS reporter gene in tobacco leaves transformed with a CaMV35S::GUS construct (35S promoter of the cauliflower mosaic virus [CaMV] fused to the β-glucuronidase gene) occurs 2 hr after bombardment. However, bombardment may be a problematic method when evaluating genes that are regulated by ethylene because it causes tissue damage, which potentially induces wounding responses that include increased ethylene production. Wound-induced ethylene production follows a distinct temporal pattern in most tissues, where ethylene production increases after an initial lag, followed by at least one additional peak (Saltveit and Dilley 1978). Consequently, the wound-induced ethylene response may be a confounding variable when studying genes regulated by ethylene.

In this study we demonstrate the stimulation of wound-induced ethylene production by particle bombardment, and discuss the importance of this response when evaluating the expression of genes that may be regulated by ethylene.

Methods

Plant Materials

Tobacco *(Nicotiana tabacum)* seeds (Lehle Seeds) were surface sterilized with 70% ethanol for 30 sec, rinsed several times with sterile deionized water, then sown on sterile soil. Plants were grown for 6 weeks under greenhouse conditions, and fertilized weekly with 0.5x Murashige and Skoog Basal Salt Mixture (MS). Seeds of *Arabidopsis thaliana* (L.) wild type Col-1 (CS3176) and transformant line CS31383 were obtained from the Arabidopsis Biological Resource Center. Surface sterilization of *Arabidopsis* seeds began with an initial ethanol wash, followed by three rinses with sterile deionized water, incubation in a mixture of 0.1% Tween 80 and commercial bleach (6% sodium hypochlorite) for 5 min, and five rinses in sterile deionized water. The seeds were planted in AIS (Enriched) Arabidopsis Growth Medium (Lehle Seeds), cold treated for 3 days at 4°C, then grown under white light from Phillips R4 fluorescent flood bulbs (75 µmol/m^2/sec) at 23–25°C or under greenhouse conditions, and fertilized weekly with 0.5x MS salts.

Particle Bombardment Conditions

Tobacco leaves were bombarded with gold particles (0.6 µm diameter) coated with 1 mg plasmid per shot using a Helios® gene gun (Bio-Rad Laboratories, Inc.) according to the Helios Gene Gun System Instruction Manual. The plasmid-coated particles were introduced at helium pressures of 0, 75, 100, 200, 300, and 400 psi.

For time-course measurements in *Arabidopsis*, a bombardment pressure of 75 psi was used for optimum DNA delivery into fully expanded *Arabidopsis* leaves (approximately 0.1 g fresh weight each) (Helenius et al. 1999).

Wounding Conditions

Bombardment-induced wounding was compared to wounding caused by slicing the newest fully expanded tobacco leaf (approximately 0.3 g fresh weight) into approximately 1 cm segments perpendicular to the midrib using a razor blade. As with tobacco, wound-induced ethylene production was determined in fully expanded *Arabidopsis* leaves (approximately 0.1 g fresh weight each) sliced into small segments.

Measurement of Ethylene Production

Bombarded plants were incubated for 50 min (in darkness) prior to ethylene analysis because wound-induced ethylene production begins after a lag of at least 20–30 min in most tissues (Saltveit and Dilley 1978). After incubation, each bombarded leaf was excised at the stem with a sharp blade, then placed into a vial that was subsequently capped with a rubber septum. Capped vials were incubated for 20 min to allow ethylene to accumulate. Any accumulated ethylene was assumed to be caused primarily by bombardment-induced wounding, since the incubation time would be too short to include ethylene production from wounding induced by tissue excision (Saltveit and Dilley 1978, Harrison 1997) . A 1 ml headspace sample collected from the vial was injected onto an alumina F1 column (0.635 cm x 0.91 m) in a gas chromatograph (Varian 3700, Varian Scientific Instruments Division) equipped with a flame ionization detector, according

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to a previously described procedure (Harrison 1997). Known amounts of an ethylene standard (Scott Specialty Gases) were analyzed to produce a standard curve for ethylene quantitation.

For the wounding treatments, the sliced tissue was placed into 2 ml shell vials, which were sealed with a rubber septum. After 25 min incubation at room temperature, a 1 ml headspace sample was analyzed by gas chromatography (as described above) to determine the basal ethylene level prior to the onset of wound-induced ethylene production. Subsequent ethylene samples were collected at 30 min intervals to evaluate the time course of wound-induced ethylene production.

Histochemical GUS Assay

Leaves were excised 30 min after bombardment and incubated at 37°C overnight in 2 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc reaction buffer, Sigma-Aldrich Co.), 1% (w/v) N,N-dimethyl formamide, 50 mM sodium phosphate buffer, pH 7.0, 0.1 mM potassium ferricyanide, 0.1 mM potassium ferrocyanide, and 1 mM EDTA (Jefferson et al. 1987), then placed in 70% ethanol for clearing and storage. Leaf images were collected by a CC-12 digital camera (Soft Imaging System Corp.) as viewed on a stereo microscope.

Results and Discussion

The pBINGUS35S plasmid containing the GUS reporter gene (with an intron) driven by the CaMV 35S promoter, was used for studies to evaluate the effect of bombardment at different helium pressures on ethylene production. Tobacco leaves were bombarded with plasmid-coated particles at helium pressures of 0, 75, 100, 200, 300, and 400 psi. A statistically significant increase in ethylene production was observed from tobacco leaves bombarded at 100, 200, or 300 psi (P = 0.004, 0.032, and 0.006, respectively, Figure 1). Lower pressure (75 psi) did not stimulate ethylene production; however, pressures below 100 psi are not considered adequate for DNA delivery into tobacco leaves (Helenius et al. 1999). They reported that
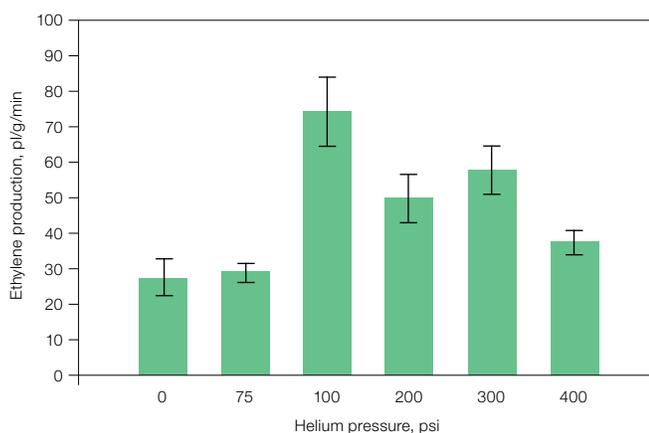

Fig. 1. Ethylene production as a result of different bombardment pressures. Tobacco leaf tissue was bombarded at different helium pressures with 0.6 μm gold particles coated with 1 μg plasmid per shot. Leaves were excised 50 min after bombardment, then placed into shell vials and sealed; headspace was analyzed for ethylene levels after 20 min incubation. N ≥4; error bars represent standard error.

200 psi helium pressure is required for optimum DNA delivery into tobacco leaves, and we observed that this level also causes wound-induced ethylene production.

A time course of ethylene production by leaves after bombardment with DNA/gold particles at a helium pressure of 200 psi was established. Measurements were performed on newly expanded leaves (approximately 0.3 g fresh weight each) still attached to the plant. Ethylene production by bombarded leaves was compared to that of leaves wounded by slicing. Tobacco leaf tissue bombarded at 200 psi helium pressure showed a wounding response similar to that of leaf tissue wounded by slicing (Figure 2). Ethylene production increased by 50 min after tissue damage, and all wounding and bombardment measurements after 50 min were significantly different than the basal level (first data point). Ethylene production by sliced tissue increased until at least 200 min after tissue damage.
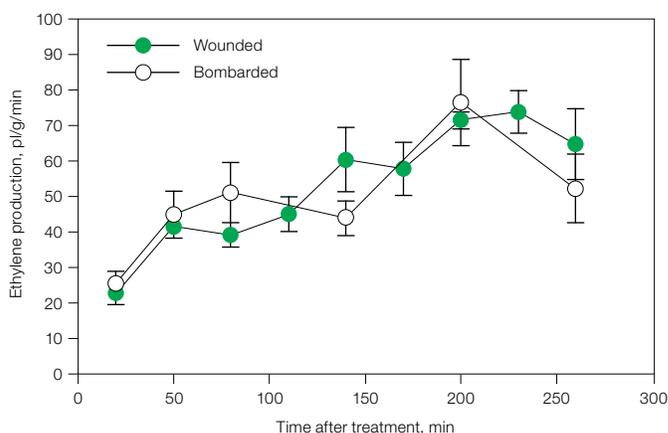

Fig. 2. Ethylene time course for wounded and bombarded tobacco leaf tissue. Leaves were wounded by cutting them into strips with a razor blade, or bombarded at a pressure of 200 psi. N ≥4; error bars represent standard error.

For *Arabidopsis*, all of the wound-induced ethylene production data points were significantly higher than the basal level (first data point) (Figure 3). The bombarded tissue also exhibited increased ethylene production for intervals 55, 85, and 115 min after bombardment, but these values were not significantly different than the basal time point. Based on these data, we conclude that although bombarding at 75 psi does cause an apparent increase in ethylene production, this increase is not statistically significant and is not at the levels observed for wounding.

Tobacco leaves bombarded with particles coated with the pBINGUS35S plasmid showed areas of normal expression in the tissue around the particles' points of entry (Figure 4A). In order to directly assess the effect of tissue wounding caused by bombardment on the production of ethylene, we evaluated the histochemical expression of wound-inducible 1-aminocyclopropane-1-carboxylate (ACC) synthase (ACS), the enzyme that regulates the primary rate-limiting step of ethylene production (Wang et al. 2002). Histochemical
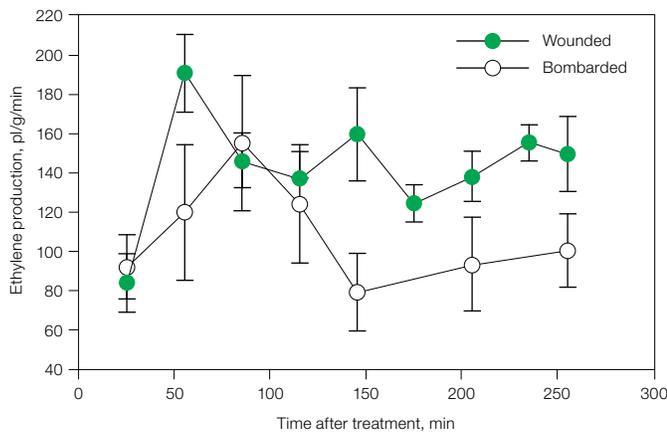
Fig. 3. Ethylene time course for wounded and bombarded *Arabidopsis* leaf tissue. Leaves were wounded by cutting them into strips with a razor blade, or bombarded at a pressure of 75 psi. N ≥4; error bars represent standard error.

analysis of ACS expression was evaluated in an *Arabidopsis* transgenic line carrying the *ACS6* promoter fused to a GUS reporter gene (developed by Tsuchisaka and Theologis 2004). When transgenic *Arabidopsis* leaves containing the *ACS6* promoter::GUS construct were bombarded with gold particles without plasmid at 75 psi, GUS expression was increased in the tissue in a reticulate pattern around the bombardment area (Figure 4B). *ACS6* is induced by any mechanical damage (Tsuchisaka and Theologis 2004), so the edge where the leaf was excised also showed strong GUS expression. These results demonstrate a wounding pattern within the bombardment area where increased ACS expression would contribute to a localized increase in ethylene production.
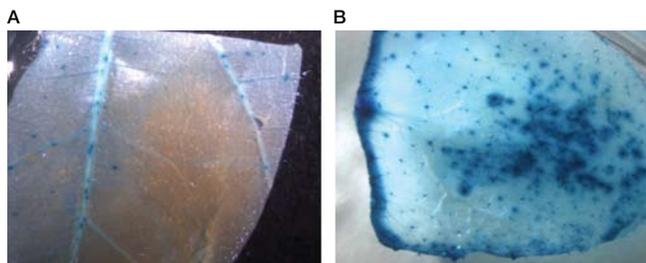
A   B

Fig. 4. Bombarded leaves. A, wild-type tobacco leaf bombarded at 200 psi with 0.6 μm gold particles coated with 1 μg pBINGUS35S plasmid shows spots of GUS expression around the sites of particle entry into the tissue; **B**, transgenic *Arabidopsis* leaf carrying *ACS6* promoter::GUS constructs bombarded with 0.6 μm gold particles (without plasmids) at 75 psi shows GUS staining throughout the bombarded site near the tip of the leaf. GUS staining also occurs at the cut edge where the leaf was excised from the plant.

Our results demonstrate that bombardment-induced ethylene production begins within 50 min after damage, and is higher than the basal level from 50 min to 2 hr after treatment (Figures 2, 3), which is within the period of highest transcription levels of genes introduced by bombardment (Miyamoto et al. 2000). Therefore, bombardment-induced ethylene production could have unexpected effects on transcript abundance for genes regulated by ethylene, thus, making it difficult to discern the promoter's ability or inability to direct transcription due to the

effect of wound-induced ethylene. Decreasing the bombardment pressure could reduce tissue damage, potentially decreasing the level of wound-induced ethylene produced, but decreased pressure may be insufficient for adequate delivery of DNA into leaf tissue (Helenius et al. 1999). It should be noted that the pattern of wound-induced ethylene production is consistent among tissues, but the level of ethylene expression varies greatly. In general, the initial wound-induced ethylene peak is the highest, after which several smaller peaks may occur, with ethylene production returning to the basal level by 4 hr after wounding (Saltveit and Dilley 1978). Therefore, strong wound-based ethylene production may only temporarily affect the expression level of ethylene-induced genes.

Microarray studies have identified numerous ethylene-regulated genes. An analysis of 6,000 unique genes in *Arabidopsis* revealed that approximately 7% were ethylene regulated (Zhong and Burns 2003). This study identified transcription factors and signaling components representing the major gene groups regulated by ethylene, including those associated with hormone regulation (auxin, cytokinin, and ethylene, since it regulates its own biosynthesis), defense, and wounding responses. An extensive *Arabidopsis* microarray analysis that examined more than 22,000 genes identified 628 genes altered by exogenous ethylene treatment (Alonso et al. 2003). Of these, 244 genes showed increased expression in the presence of ethylene, while 384 genes were down regulated. The functions of the genes were widespread, with members involved in metabolism, signal transduction, and transcriptional regulation (Zhong and Burns 2003). Given the number of ethylene-regulated genes, the role of ethylene in gene expression should be considered in transient studies and also when attempting to develop stable transformants.

In addition to direct gene regulation by ethylene, expression may also be affected by elements and/or factors located around the integration site. In a study of a minimal peach chlorophyll type II *a/b*-binding protein gene promoter developed to drive tissue-specific expression in tomato plants, different transgenic lines exhibited varied expression. The authors suggested that this could have been caused by endogenous regulatory elements near the location of integration into the plant chromosome (Bassett et al. 2007).

The regulation of a gene's expression may also be altered when the gene is inserted into a plant of a different species. An examination of the mung bean *VR-ACS1*, which has a complex expression pattern regulated by ethylene and wounding, showed that when it is inserted into tobacco or *Arabidopsis*, it is no longer regulated and acts as a strong constitutive promoter (Cazzonelli et al. 2005). Therefore, when selecting promoters for targeting expression in transgenic research, it is important to evaluate the level of regulation by the specific tissues during developmental events, such as fruit ripening, and under changing environmental conditions that are likely to cause an increase in ethylene production.

Researchers can examine expression changes after ACC (an ethylene precursor) treatment for any *Arabidopsis* gene using the Arabidopsis eFP Browser (http://bbc.botany.utoronto.ca/efp/cgi-bin/efpWeb.cgi) from The Bio-Array Resource for Arabidopsis Functional Genomics. This tool provides a user-friendly graphical representation of large sets of microarray data. The browser also presents expression information for potential orthologs between poplar and *Arabidopsis*. The identification of *Arabidopsis* gene orthologs might provide a mechanism for predicting ethylene-induced genes in other species. Another test for ethylene inducibility of a given gene would be to evaluate gene expression after incubating the target tissue in exogenous ethylene, or by determining whether ethylene production in the target tissue is induced in the presence of ACC or ethephon, an ethylene-generating compound.

Conclusions

Relative to ethylene production, bombarded tobacco leaf tissue exhibits a wound response similar to that of chopped tissue and, significantly, this wound-induced ethylene production occurs within the time period of optimum expression of genes introduced by particle bombardment. Therefore, expression studies should first determine whether the gene of interest is potentially regulated by ethylene, and whether the time course of expression falls within the range where expression would be affected by wound-induced ethylene production. Given that many genes are regulated by ethylene, understanding the potential for this confounding factor is an important design consideration when using particle bombardment or any system that wounds the tissues in order to introduce genes. For the same reason, an increase in endogenous ethylene production during development or in response to wounding or stress conditions may influence the transcript level in stable transformants of ethylene-inducible genes.

Acknowledgements

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