Rethinking how volatiles are released from plant cells

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Abstract

For plant volatile organic compounds (VOCs) to be emitted, they must cross membrane(s), the aqueous cell wall, and sometimes the cuticle, before moving into the gas phase. It is presumed that VOC movement through each barrier occurs via passive diffusion. However, VOCs, which are primarily nonpolar compounds, will preferentially partition into membranes, making diffusion into aqueous compartments slow. Using Fick's first law, we calculated that to achieve observed VOC emission rates by diffusion alone would necessitate toxic VOC levels in membranes. We propose that biological mechanisms, like those involved in trafficking other hydrophobic compounds, must contribute to VOC emission. Such parallel biological pathways would lower barrier resistances, thus steady-state emission rates could be maintained with significantly reduced intramembrane VOC concentrations.

VOCs must cross multiple cellular compartments to reach the environment

Plants emit up to 10% of photosynthetically fixed carbon in the form of volatile organic compounds (VOCs) [1]. These metabolites, when released from leaves, flowers, and fruits into the atmosphere, and from roots into the soil, play key roles in the attraction of pollinators [2] and seed dispersers [3,4], above- and below-ground defense against herbivores [5-8], protection against pathogens [9,10], and plant-plant signaling [11,12]. Certain VOCs are also able to protect plants against abiotic stresses such as high light, temperature, or oxidative stress [13-15]. While VOC functions have long been studied, the last decade has also resulted in substantial progress in the understanding of VOC biosynthesis and regulation [16]. It has been shown that VOC production and release are spatially, developmentally and/or temporally regulated and depend on biotic (e.g. pollination status and herbivore infestation) and abiotic (e.g. light intensity, atmospheric CO₂ concentration, temperature, relative humidity, and nutrient status) factors [13,16,17].

Based on their biosynthetic origin, plant VOCs can be divided into several classes, including terpenoids (monoterpenes and sesquiterpenes), phenylpropanoids/benzenoids, fatty acid derivatives and amino acid derivatives [13,16,18]. Despite such chemodiversity, plant VOCs are generally lipophilic low-molecular-weight (~100-200 Da) compounds with high vapor pressures at ambient temperature. These physicochemical properties allow VOCs to be emitted into the environment, although the site from which this occurs varies. The biosynthesis of VOCs in non-green tissues (e.g. flowers or roots) occurs predominantly in epidermal cells, which are in closest proximity to the atmosphere [19-22] or rhizosphere [23] for immediate release. In vegetative organs, VOCs are often synthesized in the secretory cells of glandular trichomes located on the leaf surface [24-27], and then secreted to a sac created by an extension of the cuticle where they are stored until mechanical disruption [28-30]. When trichomes are not involved in vegetative VOC production, these

compounds are often synthesized in mesophyll cells [31,32], mainly in the palisade parenchyma [33], and released through stomata [34-37], mechanical disruption, or emission through cuticle [36]. Regardless of the tissue and if they exit via stomata, at the subcellular level, VOCs must move from their site of biosynthesis through the cytosol to the plasma membrane, and then subsequently traverse the plasma membrane, hydrophilic cell wall, and in some cases the cuticle in order to exit the cell. While mechanical disruption provides direct access for VOCs inside the cell to the atmosphere, it still remains enigmatic how VOCs cross these barriers for release from intact cells directly to the environment or to the intercellular air spaces connected to stomata.

It is largely presumed that upon synthesis VOCs passively diffuse across cellular barriers into the environment. Many examples however exist where VOC emission rates cannot be explained by a concentration-dependent diffusion mechanism [38-41]. Assuming VOC emission is solely driven by diffusion, we have calculated the concentrations of VOCs at each cellular barrier interface needed to attain published emission rates. Based on the extremely high concentrations of VOCs predicted to accumulate in membranes, we deduce that this would be detrimental to membrane integrity and function. We thus propose that active biological mechanisms are additionally required to lower VOC concentrations in membranes to achieve the observed emission rates. Such mechanisms become even more important under stresses like herbivore attack where VOC emission is drastically increased [42] and membrane integrity is compromised systemically throughout the plant [43]. Finally, based on the cellular trafficking of other hydrophobic compounds (*i.e.* waxes and diterpenes) in plants we discuss possible biologically controlled routes for VOC export out of cells.

Diffusion-based models for VOC emission

Over the past couple of decades, VOC emission has been modeled as a diffusion process based on Fick's first law, which states that the steady state flux of any molecule is dictated by the concentration gradient and the resistance of the diffusion path (Box 1). These models have primarily been formulated at the tissue level [44-48], taking into consideration the effects of biosynthesis and environmental factors [49-53]. However, very few studies have looked at VOC movement on the microscopic scale. In their work to investigate the effect of stomatal opening on VOC emission, Niinemets and Reichstein developed a meticulously detailed model taking into account the resistance of individual cellular barriers [51,52]. Barrier resistances were evaluated based on the physicochemical properties of VOCs and structural parameters of each layer. The calculated resistances were then combined to simulate VOC emission and the total internal VOC pools inside the cell.

One concern with diffusion-based models, which has not yet been addressed, is that VOCs may accumulate to cytotoxic levels in membranes. Due to their high octanol-water partition coefficients (Box 1, $K_{o/w}$), VOCs favorably partition into hydrophobic environments. For small VOCs like isoprene, the diffusivity may be high enough that build up of high concentrations does not occur in membranes. However, for most VOCs to passively diffuse at a physiologically relevant rate into subsequent hydrophilic layers, like the cell wall, they would accumulate to very high levels in membranes. To get an idea if such levels would be biologically tolerable, we calculated the barrier concentrations required to match experimentally measured VOC emission fluxes from snapdragon (*Antirrhinum majus*) flowers (Box 1) using a similar diffusion-based model as Niinemets and Reichstein [51,52]. Our calculations revealed that the concentration of a given phenylpropanoid/benzenoid or monoterpene VOC in the plasma membrane would have to be approximately 50-120 mM to sustain its reported emission flux (Box 1, Table III). This extremely high concentration is primarily due to favorable partitioning of

VOCs into the lipid phase and the high resistance imparted by the cuticle (Box 1, Figure IB). Accumulation of lipophilic molecules in membranes increases permeability, which leads to disruption of proton and ion gradients that are responsible for intracellular pH homeostasis and the driving forces for import and export of compounds out of the cell [54]. Therefore, it is our opinion that biological mechanisms must exist to lower the barrier resistance thereby reducing the VOC concentration in membranes (Box 1). This concept logically extends to interfaces between internal membranes and the cytosol as well. Despite lower VOC emission fluxes, similar mechanisms might also occur in other plant organs, although alternative emission paths must be considered. In leaves, for example, VOCs will readily partition from cell walls into the intercellular gas spaces directly connected to sub-stomatal air spaces, which provide a pathway of lower resistance to diffusion than the cuticle.

Possible biological mechanisms involved in VOC emission

Based on analogy to intracellular trafficking of other hydrophobic compounds, like waxes and diterpenes, there are several biological mechanisms that could be involved in shuttling VOCs out of undamaged cells [55]. VOCs synthesized in the cytosol will favorably partition into both plasma membranes and subcellular membranes (Figure 1). VOCs in internal membranes could be trafficked to the plasma membrane via ER-plasma membrane contact sites or via vesicle trafficking processes associated with the ER and Golgi, *trans*-Golgi network (TGN), and/or vacuole (Figure 1). These processes would also effectively reduce the accumulation of VOCs in internal membranes. Although vesicular transport of metabolites is relatively unexplored in plants, it is already thought to play a role in the movement of cytotoxic phytochemicals [56,57], hormones [58], anti-microbial compounds [59,60], pigments [61] and cuticular wax components [62,63]. VOCs synthesized in other organelles, such as monoterpenes in plastids, could

also be delivered to the ER via interorganellar membrane hemifusion (Figure 1), a mechanism recently proposed for the transfer of lipophilic metabolites between plastids and ER [64,65]. Alternatively, VOC trafficking to the plasma membrane could be mediated by soluble carrier proteins with hydrophobic pockets capable of escorting lipophilic compounds across aqueous environments [66] (Figure 1).

The subsequent translocation of VOCs across the plasma membrane to the apoplast entails the movement of hydrophobic molecules across a lipophilic layer into an aqueous compartment. One possibility is that plasma membrane localized transporters are involved in directional export of VOCs out of the cell. Recently, it has been proposed that vesicles carrying cuticle wax components deliver their cargo to plasma membrane ATP binding cassette (ABC) transporters for export to the cell exterior [63,67]. Moreover, an *Arabidopsis thaliana* plasma membrane ABC transporter, AtABCG29, has been shown to transport the monolignol *p*-coumaryl alcohol [68], which is structurally similar and shares the same core biosynthetic pathway as the VOCs isoeugenol, eugenol, and vanillin emitted from *Petunia hybrida* flowers [69]. Remarkably, expression of *PhABCG1*, a petunia AtABCG29 homolog encoding a plasma membrane localized transporter with unknown function, is regulated by the ODORANT1 (ODO1) transcription factor [70]. Because the ODO1 transcription factor is known to control several biosynthetic genes encoding enzymes involved in synthesizing precursors for phenylpropanoid/benzenoid VOCs, it will be important to determine whether PhABCG1 exports VOCs to the cell wall. Once VOCs cross the lipophilic plasma membrane, they must traverse the hydrophilic cell wall. Although our diffusion model (Box 1) does not indicate the cell wall exerts a large resistance *per se*, small carrier proteins, such as extracellular lipid transfer proteins (LTPs), may be necessary to prevent repartitioning of VOCs into the plasma membrane (Figure 1). In plants, LTPs have recently been shown to function in the export of wax lipids and diterpenes from the plasma membrane, across the cell wall, to the cuticle [71-

74]. Since LTPs generally lack substrate specificity, it is possible that the same LTPs may also be transporting VOCs to neighboring cells, intercellular spaces (in some cases for release via stomata), or the cuticle. The concentration of VOCs in the cuticle and their rate of release into the environment are dependent on the cuticle composition and its thickness [40, 75-77]. This is consistent with our calculations showing that the cuticle imposes the largest resistance to VOC emission (Box 1, Figure 1B). In the presence of biological mechanisms at the other cellular barriers, the control of the cuticle on emission will be even higher.

Concluding Remarks

In order for VOCs to be released from intact cells into the environment they must cross several subcellular barriers, each providing resistance to the overall emission rate. Examination of existing diffusion models strongly suggests that to drive observed emission rates high VOC levels would need to accumulate in the hydrophobic compartments that these molecules must traverse. Although we cannot exclude the possibility that VOC emission occurs via a solely diffusion-dependent process, it appears unlikely that this would be the case given the detrimental effects of high VOC concentrations in membranes. Therefore, it seems more plausible that there are biological mechanisms in place to facilitate emission flux with reduced VOC membrane levels. In the presence of biological control, the movement of VOCs through different barriers is analogous to current flow through circuits with parallel resistances, $r_{biological}$ or $r_{diffusive}$, in which the current will flow preferentially through the path of least resistance. In light of recent progress made in both deep sequencing technologies and understanding of intracellular transport of other hydrophobic compounds, we propose future research should explore if similar mechanisms are involved in subcellular VOC trafficking. Since the cuticle imposes the largest resistance to VOC emission, another important area of

research that should be further investigated using genetic approaches is the impact of cuticle composition on VOC emission.

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Disclaimer statement

The authors declare there is no conflict of interest.

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Figure legend

Figure 1. Proposed models for VOC trafficking in plant cells. VOCs may diffuse through each cell barrier, or be trafficked via unknown biological mechanisms similar to that of other hydrophobic compounds [55-74]. The physicochemical parameters of most VOCs likely preclude their movement through the cell solely by diffusion. Possible vesicle tracking routes for transport through the cytosol are depicted by gray block arrows. Lipid transfer proteins (LTPs) or other types of carrier proteins may contribute to shuttling VOCs through the plastid stroma, cytosol, and/or cell wall. Transporters localized in the plasma membrane may play a role in exporting VOCs from the cytosol or membrane into the cell wall. VOCs then diffuse through the cuticle for emission into the environment. The involvement of other biological mechanisms should not be excluded. Abbreviations: GPI, glycosylphosphatidylinositol.

Box 1. VOC diffusion at the subcellular level according to Fick's first law

At the subcellular level, VOCs synthesized in the cytosol must cross the plasma membrane and cell wall barriers, and in some cases the cuticle, to be emitted (Figure

IA). If diffusion is the sole driving force of VOC emission, then under steady-state conditions, emission flux (J) can be modeled by Fick's first law as (equation I):

$$J = \frac{\Delta C}{r_i}$$

where ΔC represents the concentration difference along the diffusion path and r_i is the resistance to diffusion through a given barrier, i (Figure I). The resistances through each barrier are a function of length, l_i , and VOC physicochemical properties, which include diffusivity through a given barrier, D_i , and octanol-water ($K_{o/w}$) and cuticle-water ($K_{c/w}$) partition coefficients, (ratios of concentrations of a compound in the octanol phase and cuticle, respectively, to its equilibrium aqueous-phase concentration) as follows (equations II).

$$r_{PM} = \frac{l_{PM}}{D_{PM} \cdot K_{o/w}} \quad r_{CW} = \frac{l_{CW}}{D_{CW}} \quad r_{Cut} = \frac{l_{Cut}}{D_{Cut}}$$

$$K_{o/w} = \frac{C_{PM1}}{C_{Cyt}} = \frac{C_{PM2}}{C_{CW1}} \quad K_{c/w} = \frac{C_{Cut1}}{C_{CW2}}$$

To determine the biological feasibility of VOC emission being driven solely by diffusion, we investigated the VOC concentrations in each subcellular barrier required to match experimentally measured VOC emission fluxes from snapdragon flowers [40,78]. For illustrative purposes, we modeled diffusion of a representative benzenoid/phenylpropanoid (methylbenzoate) and terpenoid (nerolidol) VOC, both of which are synthesized in cytosol [21,78]. Thus, internal membrane barrier resistances (e.g. plastid) were not considered. Since flowers lack stomata, and VOC biosynthesis occurs in epidermal cells, VOC fluxes into intercellular air spaces were not factored. Barrier resistances were calculated using equation II with parameters available from the literature (Tables I and II). Concentrations in each barrier were solved assuming uniform VOC concentrations in the cytosol and neglecting the bulk concentration (C_{bulk}) of VOCs at the air-cuticle interface. Combining these assumptions with steady-state mass

balance at each barrier interface, we solved for VOC concentrations as follows (equation III):

$$J_{syn} = \frac{(C_{Cyt} - C_{CW1})}{r_{PM}} = \frac{(C_{CW1} - C_{CW2})}{r_{CW}} = \frac{C_{Cut1}}{r_{Cut}} = J_{emi}$$

Simulation shows that a uniform VOC concentration exists in the plasma membrane as a result of its short path length and/or the high diffusivity of VOCs in this layer. Our calculations further indicate that to match measured emission fluxes [40,78], methylbenzoate and nerolidol would need to hyper-accumulate in the plasma membrane (Figure IB, Table III) to levels that are likely detrimental to the cell. Therefore, we propose that the concentration of VOCs in cellular membranes has to be lowered by biological mechanisms (Figure 1). Our simulation also indicates that the resistance imposed by the cuticle is much higher than that of the plasma membrane and cell wall, suggesting that it serves as the rate-limiting barrier to VOC emission.

Box Figure Legend and Tables

Figure I. (A) Diagram depicting VOC emission barriers in snapdragon flowers. **(B)** Simulated VOC concentrations (equation III) within each barrier needed to match measured emission rates of methylbenzoate (black line) [40] and nerolidol (blue line) [78] solely by passive diffusion. See Table III for calculated concentration values.

Table I: VOC physicochemical properties and fluxes

VOC Property	Symbol (Unit)	Value ^a [Reference]	
		Methylbenzoate	Nerolidol
Diffusivity in cuticle ^b	$D_{Cut} (m^2 s^{-1})$	10^{-16} [79]	10^{-17} [80]
Diffusivity in cell wall	$D_{CW} (m^2 s^{-1})$	7×10^{-10} [81]	5×10^{-10} [81]
Diffusivity in plasma membrane ^b	$D_{PM} (m^2 s^{-1})$	2×10^{-10} [82]	10^{-7} [82]
Octanol-water partition coefficient	$K_{o/w}$	$10^{2.1}$ [83]	$10^{5.3}$ [84]
Cuticle-water partition coefficient ^b	$K_{c/w}$	$10^{1.1}$ [79]	$10^{4.3}$ [79]
Emission Flux	$J_{emi} (mol m^{-2} s^{-1})$	7.5×10^{-9} [40]	3×10^{-10} [78]

^aaccurate to within an order of magnitude

^bdetermined using empirical relations developed in respective references

Table II: Tissue structural properties

Characteristic	Symbol (Unit)	Value (Reference)
Cuticle diffusion path length	$l_{Cut} (m)$	1.6×10^{-7} [40]
Cell wall diffusion path length	$l_{CW} (m)$	4×10^{-7} [40]
Plasma membrane thickness	$l_{PM} (m)$	10^{-8} [85]
Total petal area	$A_p (m^2)$	7×10^{-4} [40]

Table III: VOC concentrations in each barrier required to match emission flux solely by passive diffusion

Barrier	Concentration (C)			
	Methylbenzoate		Nerolidol	
	C_1	C_2	C_1	C_2
Cytosol	0.95 mM	n/a	0.24 μ M	n/a
Plasma membrane	120 mM	120 mM	48 mM	48 mM
Cell wall	0.95 mM	0.95 mM	0.24 μ M	0.24 μ M
Cuticle	12 mM	0	4.8 mM	0

Figure 1