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# Proteomic analysis of secreted saliva from Russian Wheat Aphid (*Diuraphis noxia* Kurd.) biotypes that differ in virulence to wheat

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ABSTRACT

*Diuraphis noxia*, Russian Wheat Aphid (RWA), biotypes are classified by their differential virulence to wheat varieties containing resistance genes. RWA salivary proteins, unlike those of most aphid species, cause foliar damage and physiological alterations in plants. A comparative proteomic analysis of secreted saliva from four differentially virulent RWA biotypes identified thirty-four individual proteins. The five major proteins were glucose dehydrogenase, lipophorin, chitinase, CiV16.8g1-like, and lava lamp. Fourteen proteins quantitatively varied among biotypes; trehalase, β-N-acetylglucosaminidase (chitinase), two separate glucose dehydrogenases, calreticulin, aminopeptidase, acetylglucosaminyltransferase, hydroxymethylglutaryl-CoA lyase, acyltransferase, ficolin-3, lava lamp, retinaldehyde-binding protein, and two proteins of unknown function. Fifty-four percent of spectral counts were associated with glucose dehydrogenase, which is thought to detoxify plant defensive compounds. One-dimensional electrophoresis detected nine protein bands from 9 to 60 kDa that quantitatively differed. Two-dimensional electrophoresis identified six major gel zones with quantitative and qualitative variance in proteins. Our findings reveal that the salivary proteome of RWA, a phytotoxic aphid, differs considerably from those reported for nonphytotoxic aphids. The potential roles of proteins used in the general plant feeding processes of aphids and those that are potential phytotoxins related to aphid virulence are discussed.

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## 1. Introduction

Plants have evolved a variety of complex mechanisms to prevent herbivory and its related effects on plant health. Primary defensive mechanisms include constitutive defenses such as mechanical adaptations (e.g. trichomes, thorns, lignins, silicates) and the production of allelochemicals (e.g. alkaloids, phenols, proteins) aimed at discouraging or preventing herbivory [1]. Herbivore attack often elicits the initiation of induced plant defensive pathways (e.g. ethylene, ROS, and salicylic and jasmonic acid pathways) triggered either by wounding or by direct interactions between plant and insect proteins [1–3]. The induction of plant defense responses as a result of herbivory leads to the expression of pathogenesis-related (PR) and defensive proteins that typically serve to limit the spread of a pest or pathogen [2,4–6]. Aphid attack induces defensive protein expression in the plant host [2,6–8], but the initial response of upregulating plant defensive genes is not sustained and exhibits reduced expression as

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the aphid infestation continues [8,9]. This phenomenon suggests that there are other underlying mechanisms involved in plant defenses against herbivore attack, especially with regard to aphid–plant interactions.

Aphids have evolved an intricate relationship with plants by suppressing or subverting host plant defenses in order for the aphid to establish phloem sap feeding and modify the nutritional value of the phloem [10–15]. The aphid's specialized hollow needle-like mouthparts (stylets) and salivary glands are fundamental to this relationship. Physical plant injury or puncturing of phloem sieve elements normally leads to rapid phloem sieve element occlusion involving the formation of vesicles and insoluble protein complexes within the sieve elements that accumulate at sieve element junctions, effectively occluding the sieve element [10,16]. Phloem occlusion and related defensive responses are avoided by the aphid during feeding through direct interaction of its salivary proteins with host plant proteins and elicitors [10,11]. Saliva is secreted continually as the aphid's stylet penetrates the plant leaf surface and is steered through the plant tissues to the sieve elements of the phloem. There are two primary types of saliva that serve separate functions during the feeding process. Gelatinous saliva is known to contain a mixture of proteins (e.g. phenoloxidases, glucosidases) and other materials which facilitate plant penetration and forms a semi-rigid insoluble sheath around the stylet as it penetrates the plant [12,17–19]. The sheath creates a barrier between the stylet and the plant to prevent aphid contact with the plant and the subsequent activation of plant defenses. It also acts to form a leak-proof seal to assist in stylet tube function, and is used to plug the sheath tube when the aphid removes the stylet upon exiting the plant [12]. Watery saliva is a complex mixture of amino acids, proteins (e.g. hydrolases, oxidases) and other materials that the aphid uses to modify plant defenses and phloem chemistry in order to successfully feed on the phloem [10,12,13,15,19–21]. The aphid's watery saliva contains a wide range of protein classes with distinct functions [13–15,21,22] which act in concert to allow the aphid to continue feeding while avoiding further elicitation of plant defenses.

The majority of aphid species typically cause little or no observable damage to the plant under low numbers and may be considered "nonphytotoxic" (e.g. *Acyrthosiphon pisum* (Harris), *Myzus persicae* (Sulzer)). Nonphytotoxic aphids cause, at most, minor distortions of the leaf and produce copious quantities of excreta (honeydew). In contrast, there are some aphids that, even in low numbers, cause severe visible damage to plants including necrosis and chlorosis (e.g. *Schizaphis graminum* (Rond.)), or chlorotic interveinal streaking and leaf rolling (*Diuraphis noxia* (Kurdjumov)), and the damage often is systemic [12]. In general, aphid groups can be regarded as nonphytotoxic or phytotoxic aphid models, where the latter can be further classified as virulent or avirulent to specific plant genotypes [23,24].

*D. noxia*, commonly known as the Russian Wheat Aphid (RWA), is a phytotoxic aphid and a significant pest of wheat and barley in the United States and South Africa. Plant damage resulting from RWA feeding consists of leaf rolling, longitudinal leaf chlorosis, and reduced root and tiller development with concomitant plant stunting and death, all of which ultimately reduces grain yields [25,26]. Nine resistance genes (*Dn*1–9) have been identified to manage RWA through development of resistant wheat cultivars. This pest was successfully managed from 1995 to 2002 by the use of resistant wheat carrying the *Dn*4 gene, but in 2003 a new RWA biotype emerged (RWA2) that was virulent to all *Dn* genes except *Dn*7 [27]. Additional RWA biotypes (RWA3–8) have been identified in the field that exhibit differential virulence patterns to the nine resistance genes [28]. *D. noxia* can continue to feed and reproduce on resistant varieties despite the initial upregulation of defensive responses in infested resistant and susceptible wheat genotypes [6–8] even although the characteristic plant damage symptoms in resistant genotypes are mitigated. Currently, the occurrence of RWA biotypes poses a significant challenge to the development of new resistant wheat cultivars.

Knowledge of the salivary constituents and a comparison of these components among RWA biotypes would lead to better understanding in how *D. noxia* damages wheat and overcomes resistance mechanisms. Herein, we present detailed 1-D and 2-D gel electrophoresis and LC–MS/MS analyses that compare the salivary proteomes of *D. noxia* biotypes RWA 1, 2, 5, and 8 to identify proteins that are commonly expressed or variably expressed. The selected RWA biotypes represent high (RWA2), intermediate (RWA5), and low (RWA1 and 8) virulence as measured by the number of RWA resistance genes they are able to overcome [28–30]. The resulting peptide data and analyses were used to search EST databases of the Pea Aphid, *A. pisum* and the Green Peach Aphid, *M. persicae*, and an Arthropoda protein database, to arrive at protein identifications and functions.

## 2. Materials and methods

### 2.1. *Aphid growth and maintenance, saliva collection and preparation*

RWA biotypes 1, 2, 5, and 8, were obtained from the USDA-ARS Cereal Insect Genetic Resource Library (CIGRL) and reared under standard conditions (22 °C, 16:8 Light:Dark) in growth chambers on winter wheat (cultivar TAM110) in 16 cm pots. Plants and aphids were caged by 14 cm diameter clear polycarbonate cylinders 35 cm tall topped with fine mesh nylon screen to confine the aphids and prevent cross-contamination. When aphids reached approximately 5000/pot, and host plants displayed less than 30% foliar damage according to a commonly used nine-point damage rating scale [31], the aphids were removed by gentle shaking to reduce aphid injury. Aphids were collected on white paper, cleaned of debris, weighed, and then placed on a diet of 15% sucrose (weight/volume, prepared with molecular biology-grade water) sealed in stretched parafilm on bottoms of 15 cm diameter Petri dishes [15]. Approximately 12,000 aphids per dish were allowed to feed for 24 h. The 15% sucrose diet was collected by gently peeling back a small portion of the parafilm covering and slowly pipetting out the free-flowing liquid. Diet not flowing freely from the plates was not collected to avoid potential contamination. As a negative control, an equal number of uninfested 15% sucrose plates were held for the same period of time and diet was collected. The collected diet (ca. 10 dishes with a total of 120,000 aphids per saliva collection) was placed immediately in pre-chilled Vivaspin 20