Research Article

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Seed priming with calcium chloride enhances wheat resistance against wheat aphid *Schizaphis graminum* Rondani

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Abstract

BACKGROUND: Calcium is an essential macronutrient for plant growth. Although it has been shown that exogenous Ca application can increase plant resistance to abiotic stress, little is known about its potential to enhance plant tolerance to biotic stress. Here, we investigated whether pretreatment of wheat (*Triticum aestivum* L.) seeds with calcium chloride ($CaCl_2$) improves plant resistance against wheat aphid (*Schizaphis graminum* Rondani). The developmental time, population size, feeding behavior of aphids on plants grown from $CaCl_2$- and water-pretreated seeds, and plant defense responses to aphid attack were investigated.

RESULTS: Seed pretreatment with $CaCl_2$ extended aphid development time and reduced aphid population size and feeding efficiency. In addition, the pretreatment significantly increased the concentration of Ca^{2+} in wheat leaves, and upregulated expression levels of *TaCaM* genes and callose synthase genes (*TaGSL2*, *TaGSL8*, *TaGSL10*, *TaGSL12*, *TaGSL19*, *TaGSL22* and *TaGSL23*). Callose concentration in the leaves of plants grown from $CaCl_2$-pretreated seeds increased significantly upon aphid attack. Further, callose deposition was observed mainly in the phloem.

CONCLUSION: These results suggest that seed pretreatment with $CaCl_2$ primes the plant response against wheat aphid attack, leading to modulation of callose deposition in the phloem in response to aphid attack.

Supporting information may be found in the online version of this article.

Keywords: seed priming; herbivore resistance; calcium; callose; wheat aphid (*Schizaphis graminum* Rondani); wheat (*Triticum aestivum* L.)

1 INTRODUCTION

Wheat (*Triticum aestivum* L.) is an important food crop grown worldwide and used as a staple food in many countries.[1] The wheat aphid (*Schizaphis graminum* Rondani) (Hemiptera: Aphididae) is a destructive insect pest of many cereal crops and causes severe damage by phloem sap ingestion and virus transmission.[2–5] However, efforts to control this pest by spraying crops with high doses of multiple pesticides pose serious risks to the environment and have resulted in pesticide resistance.[6–8] Therefore, continued efforts are required to explore more effective and environmentally friendly methods to control wheat aphid. Host plant resistance has been demonstrated to be effective in managing insect pests in many crops.

Plant defense priming is a physiological process, whereby plants prepare to respond to imminent stress more quickly or more aggressively.[9–13] Priming is initiated after a plant is exposed to an environmental cue or stress that reliably shows an increased probability of encountering an enemy. Recent evidence suggests that priming can be an effective tool for crop protection in the field.[14–16] Seed priming is used to improve germination by managing a series of parameters during the initial stages of plant development. An array of studies show that seedlings that emerged from primed seeds displayed earlier and more uniform germination, and robust and prompt cellular defense responses against abiotic stress.[9, 17–20] Worrall *et al*. demonstrated that

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anti-herbivore defense priming could be elicited by pre-imbibition of seeds with 3.0 mM jasmonic acid (JA) in tomato (*Solanum lycopersicum* cv. Carousel), which conferred significantly enhanced resistance against tobacco hornworms (*Manduca sexta*), green peach aphids (*Myzus persicae*) and spider mites (*Tetranychus urticae*).[21] Similar results were also obtained with Norway spruce (*Picea abies*) seedlings grown from seeds pre-imbibed with 3.0 mM JA, which exhibited a 62.5% reduction in pine weevil (*Hylobius abietis*) infestations relative to seedlings grown from untreated seeds.[22] Such seed treatments could offer obvious economic advantages over within-crop spray treatments without detectable reduction in growth. Moreover, such an approach could easily be applied by seed distributors and require no additional labor input from growers.

Calcium is an essential element for plants, and numerous studies have shown that calcium-containing compounds, including $Ca(NO_3)_2$, $Ca(OH)_2$, $CaCO_3$ and $CaCl_2$, can confer on crop plants resistance to abiotic and biotic stresses, for example, seed priming with $CaCl_2$ could alleviate salinity-induced stress in rice[23] and improve drought resistance in maize.[24] However, the induction effect of Ca to biotic stresses mainly focused on plants resistance against disease, including bacterial wilt and powdery mildew in tomato,[25, 26] bacterial wilt in tobacco,[27] leaf blast in wheat,[28] *Sclerotinia sclerotiorum* and phytophthora stem rot in soybean.[29, 30] The effects of Ca on plants resistance to insects are largely unknown. Our previous study showed that kidney bean plants treated with different concentrations of $CaCl_2$ solution using leaf spraying, root irrigation or seed soaking all had enhanced lipoxygenase, phenylalanine ammonia lyase and β-1,3-glucanase activities in leaves inoculated with the insect *Frankliniella occidentalis* (Thysanoptera: Thripidae). Furthermore, the developmental time of the whole immature stage of *F. occidentalis* fed on plants sprayed with 10 mM $CaCl_2$ was longer than on control plants.[31] However, whether exogenous $CaCl_2$ could enhance anti-herbivore resistance in monocotyledonous plants such as wheat against insect pests needs further research.

Plants depend on Ca signals to rigorously regulate immune responses.[32, 33] When plants are subjected to various abiotic and biotic stresses, cell membrane receptors receive a signal and undergo a series of phosphorylation reactions that can cause Ca^{2+} to flow into the cytoplasm. As the concentration of Ca^{2+} in the cytoplasm increases, a calcium signal with temporal and spatial specificity is formed within a short timeframe.[34–36] Reactive oxygen species, electrical signals and changes in cytosolic Ca^{2+} concentrations ($[Ca^{2+}]$cyt) are thought to form signaling networks supporting both local and systemic plant defense responses.[36–38] Further transmission of Ca signals activates JA, salicylic acid, ethylene and other signal transduction pathways, thereafter inducing plant defense responses.[39–42]

Callose is a natural permeable barrier and a leak-seal in plant tissues injured by herbivores.[43–46] Particularly in response to phloem-feeding insects, callose closure of the sieve pores and callose coagulation on the sieve plates serve as a physical barrier to prevent insects from consuming the phloem sap.[46–49] Studies have shown that a Ca^{2+} signal is involved in the regulation of callose synthesis.[50, 51] In addition, β-1,3-glucan callose is synthesized by glucan synthase-like (GSL) enzymes, and deposits on sieve plates.[52] Previous studies have demonstrated that seven *TaGSL* genes (*TaGSL2*, *TaGSL8*, *TaGSL10*, *TaGSL12*, *TaGSL19*, *TaGSL22* and *TaGSL23*) are highly expressed in wheat leaves.[53] Repression of phloem-feeding activity is closely associated with increased callose deposition and enhanced expression of *TaGSL* genes (*TaGSL2*, *TaGSL10* and *TaGSL12*).[54–56]

Activation of primed resistance by chemicals in seeds may provide a simple and feasible way of achieving long-term improvements in stress resistance in crop plants. Guizi 1 (GZ1, Certificate No. Qian, 2 015 003) is a stable and high-yielding purple grain wheat variety. In this study, we investigated whether seed pretreatment with $CaCl_2$ increased wheat resistance against wheat aphid *Schizaphis graminum*. The results are of great significance to promote the pollution-free green production of this purple grain wheat.

2 MATERIALS AND METHODS

2.1 Plant treatments

Seeds of the wheat variety GZ1 were sterilized in 10% hydrogen peroxide for 10 min and then rinsed three times with deionized water. Seeds were soaked in an excess of 20 mM $CaCl_2$ or deionized water (control) for 2 days and subsequently germinated for 2 days at 25°C. Upon shoot protrusion, seeds were planted in plastic pots (two or three seeds/pot, pot diameter: 12 cm, height: 15 cm) filled to two-thirds of the pot volume with the soil mixture (soil/substrate = 1:1), and the newly emerged seedlings were then covered with the soil mixture to complete the remaining one-third of the volume. Plants were maintained in a growth chamber with a day/night temperature regime of 25 ± 2°C, a 14:10 h light/dark photoperiod and 60% relative humidity (RH). Plants were used in the experiments 20 days after planting.

2.2 Insects

Wheat aphids were originally obtained from GZ1 wheat plants in the Guizhou Branch of the National Wheat Improvement Center in China. Aphids were maintained on GZ1 wheat plants in a climate-controlled room (25 ± 2°C, 60% RH and a 14:10 h light/dark photoperiod). The population was continuously reared for more than 30 generations in the laboratory. Nymphs (24 h old) and wingless parthenogenetic female adult aphids were used for feeding treatments and bioassays, respectively.

2.3 Aphid performance bioassay

Plants grown from seeds treated with $CaCl_2$ and from seeds treated with water only (controls) were inoculated with one 10-day-old wingless parthenogenetic female adult aphid on the adaxial surface of the second unfolding leaf in each plant (one plant/pot). Aphids were confined to individual plants using a tubular polycarbonate plastic cage (10 cm in diameter × 40 cm high) with organdy fabric secured by rubber bands. To facilitate observation, 24 h after aphids were applied to the plants, we removed nymphs with a brush, leaving only one nymph aphid on each plant. The nymph development rate until adulthood was observed in an artificial climate chamber under the following conditions: 25°C constant temperature, 14:10 h light/dark photoperiod and 60% RH. Thirty replicates were included for each treatment.

For bioassays of the aphid population, plants grown from seeds treated with $CaCl_2$ and plants grown from seeds treated only with water were inoculated with ten 10-day-old wingless parthenogenetic female adult aphids. After 24 h, all adult aphids were removed, and only five aphid nymphs remained on each plant. The number of aphids settling on the leaves was recorded on day 10. Fifteen replicates were included for each treatment.

2.4 Aphid feeding behavior

The feeding behavior of wingless parthenogenetic female adult aphids was monitored using the Giga-8 DC-EPG system

(Wageningen University, the Netherlands).[56] Ten-day-old wingless parthenogenetic female adult aphids were starved for 2 h before experiments and then placed on the second unfolding leaf of 20-day-old plants. A gold wire (10 μm in diameter and 2–3 cm in length) was attached to the dorsum of each aphid using water-based conductive silver glue, and wires were connected to an electrical penetration graph (EPG) insect electrode. The other electrode was inserted in the soil of each potted plant. The feeding activities of the aphids were recorded for wheat seedlings grown from seeds pretreated with $CaCl_2$ or with water. Plant, insect and EPG probes were maintained inside a Faraday cage and monitored continuously for 6 h. Each aphid and each plant were used only once until 20 successful replicates for each treatment were obtained. Data were recorded and analyzed with Stylet + software (W.F. Tjallingii, Wageningen, the Netherlands) and the stylet pathway waveforms were distinguished according to Tjallingii.[57] EPG parameters were calculated using the Excel workbook for automatic parameter calculation of EPG data 4.3.[58]

2.5 Callose deposition

Ten-day-old wingless, parthenogenetic female, adult aphid females were transferred into a sachet (3 × 3 cm) and fastened to the second leaves of a 20-day-old seedling.[49] The aphids were left to feed on the plants for 24, 48 and 72 h before the sachet was removed and the plant segments damaged by the aphids were collected. Undamaged plants were sampled in the same manner. Aphid-damaged leaves (0.2 cm long) were covered with an embedding matrix to obtain frozen sections (Cellpath) and cut into 15-μm sections using a frozen tissue microtome (Leica CM1950). More than 20 cross-sections were obtained from the leaves of each plant. Glass slides were left to dry at room temperature for 1 h, to ensure the sections and the glass slide were completely glued together. Glass slides were then soaked in deionized water to completely dissolve the embedding matrix solution from the frozen tissue sections. Subsequently, the leaves were dehydrated with 95% ethanol for 3–5 h, and the residual ethanol on the slides was removed using deionized water. Leaf samples were stained with 0.1% (w/v) aniline blue dye, and then kept in a dark environment for 12 h before rinsing with deionized water and drying in a dust-free area. Finally, dried sections were observed under ultraviolet (UV) light using a fluorescence microscope (Nikon 80i), and recorded photographically. The images were analyzed with Image Pro-plus 6.0. Three replicate assays were performed for these experiments, and three samples from independent wheat plants were included in all treatments for each replicate.

2.6 Measurement of Ca^{2+} and callose concentration

Aphid treatments were the same as described in Section 2.5. Leaf Ca^{2+} and callose concentrations were measured using a plant Ca^{2+} enzyme-linked immunosorbent assay (ELISA) kit and a callose ELISA kit (catalog numbers: JL45982 and JL46407; Shanghai Jianglai Industrial), respectively. Assays were conducted according to the manufacturer's protocols. The detection ranges for callose and Ca^{2+} were 2.5–80 ng ml^{-1} and 9.375–300 pg ml^{-1}, respectively.

2.7 Gene expression analysis

Differential expression of selected genes was determined using real-time quantitative reverse transcription polymerase chain reaction (qRT–PCR). Total RNA was extracted using the Eastep® Super Total RNA Extraction kit (Shanghai Promega). First-strand complementary DNA (cDNA) was synthesized from 1 μg of total RNA using HiScript® II Q RT Super Mix for qPCR (Vazyme), and qRT–PCR was performed using ChamQ Universal SYBR qPCR Master Mix (Vazyme), according to the manufacturer's instructions. RNA samples were reverse transcribed into cDNAs and used as templates to perform qRT–PCR in a C1000 Thermal Cycler (Bio-Rad). The wheat housekeeping gene *18SrRNA* (GenBank accession no. AY357914) was used as an endogenous control for sample normalization. The gene-specific primer sequences used are listed in Table S1. Real-time PCR reactions were carried out with 0.8 μl of upstream and downstream primers, 2 μl of cDNA and 10 μl of SYBR Green Master Mix. The final volume was adjusted to 20 μl with double-distilled water (ddH_2O). Reaction conditions for thermal cycling were as follows: 95°C for 5 min, 40 cycles at 95°C for 10 s, 55°C for 30 s and 72°C for 30 s. The fluorescence signal was collected during the cycle at 72°C. Amplicon specificity was verified by melting curve analysis and agarose gel electrophoresis. The relative expression levels for targeted genes were calculated using the $2^{-\triangle\triangle Ct}$ method. Three replicates of each treatment were used in the assays. Finally, gene expression levels in water-pretreated plants were used as controls.

2.8 Statistical analysis

SPSS 21.0 for Windows was used for statistical analyses. Differences in aphid development time, aphid population sizes and gene expression levels at 0, 24, 48 and 72 h, between plants exposed to aphids and unexposed plants were evaluated using Student's *t*-test. Any behavioral EPG variables that did not follow a normal distribution were analyzed using the Mann–Whitney *U*-test.[59]

3 RESULTS

3.1 Aphid development on wheat plants grown from $CaCl_2$-pretreated seeds

Seed pretreatment with $CaCl_2$ significantly increased wheat resistance against aphid attack ($P < 0.05$) (Table 1). Development time for first-, second- and fourth-instar aphids feeding on plants

Table 1. Effect of $CaCl_2$ on the developmental time of *Schizaphis graminum* (Rondani)

	Time (days)				
Treatment	First instar	Second instar	Third instar	Fourth instar	Total
Control	1.47 ± 0.05	1.36 ± 0.05	1.55 ± 0.06	1.53 ± 0.08	5.92 ± 0.12
20 mM $CaCl_2$	2.00 ± 0.06**	1.72 ± 0.10**	1.65 ± 0.05	1.80 ± 0.07*	7.17 ± 0.15**

Rate of development and population size of wheat aphids (*Schizaphis graminum*) feeding on wheat plants (*Triticum aestivum*). Values are means ± SE ($n = 30$). Asterisks indicate significant differences in development times for number of aphids per plant grown from seeds with $CaCl_2$ pretreatment compared with plants grown from seeds treated with water (controls) (*$P < 0.05$, **$P < 0.01$, Student's *t*-test).

grown from $CaCl_2$-pretreated seeds was 36.05%, 26.47% and 17.65% longer compared with aphids feeding on control plants. The total development time of aphids feeding on plants grown from $CaCl_2$-pretreated seeds was 1.25 days longer than that of aphids feeding on control plants (Table 1). Similar to the observed longer development times, after 10 days of treatment, the average number of aphids was 29.02% lower on plants grown from $CaCl_2$-pretreated seeds than on control plants (Figure 1).

3.2 Probing and feeding behavior of aphids fed on $CaCl_2$-pretreated plants

Six hours after aphids were applied to the experimental plants, seven waveforms were identified during aphid probing, namely, np, C, pd, F, G, E1 and E2 (for details, see Table 2). Aphids feeding on plants grown from $CaCl_2$-pretreated seeds behaved differently from aphids feeding on control plants, concerning several EPG variables related to general probing behavior, namely in the pathway phase, derailed stylet mechanics, xylem ingestion and phloem phase (Table 2). The $CaCl_2$ seed pretreatment led to an increased total number of aphid probings, prolonged number of np and total duration of np, and reduced total aphid probing times (Table 2, variables 1–4). In addition, seeds pretreated with $CaCl_2$ prolonged the number and duration of C waves and produced more F waves during the process of aphid probing (Table 2, variables 6–13). No differences between treatments were observed in the behavior of aphids while feeding on the xylem, but the $CaCl_2$ pretreatment reduced the number and total duration of G waves (Table 2, variables 14 and 15). In the phloem-feeding stage, $CaCl_2$ pretreatment delayed aphid feeding (total duration of E), and increased the number of E1 and E2 waves. This result indicates that $CaCl_2$ pretreatment effectively prolonged the time needed for aphid mouth needles to secrete saliva into the phloem sieve tubes. Furthermore, $CaCl_2$ pretreatment shortened the feeding time, and significantly increased the difficulty aphids experienced while trying to feed on the phloem ($P < 0.05$) (Table 2, variables 16–24).

Compared with the control treatment, the behavior of aphids feeding on plants grown from $CaCl_2$-pretreated seeds was predominately by C-wave, whereas phloem-feeding was the dominant feeding technique used on control plants (Figure 2). $CaCl_2$ treatment resulted in a significantly higher percentage of time spent by aphids in entering the pathway phase (62.21% in the $CaCl_2$ pretreatment group and 36.72% in the control group, see Figure 2, % C). Furthermore, the percentage of time aphids spent feeding on the phloem was lower in the $CaCl_2$ pretreatment group than in the control group (32.95% and 60.25%, respectively, see Figure 2, % E2). The percentage of time spent by aphids feeding on plants using derailed stylet mechanics was 3.47% in plants grown from $CaCl_2$-pretreated seed but only 0.2% in control plants (Figure 2, % F). Additionally, there were no significant differences between treatments in the percentage of time spent on variables E1 and G (Figure 2, % E1 and G).

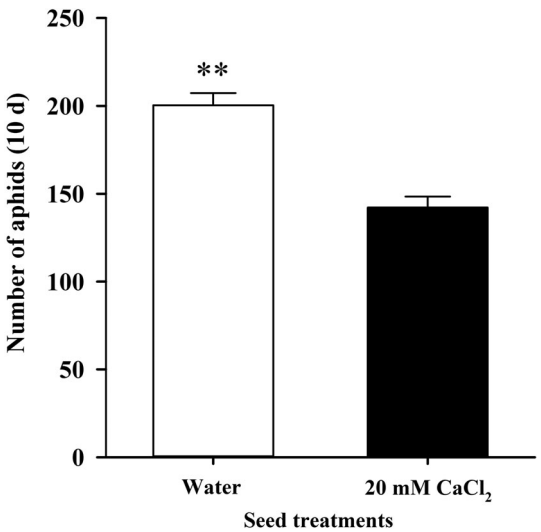

Figure 1. Populations of wheat aphids after 10 days of feeding on wheat plants grown from seeds pretreated with $CaCl_2$ or water (controls). Wheat seeds were soaked in 20 mM $CaCl_2$ or water for 48 h, and the test was conducted 20 days after planting. Values are means ± SE ($n = 15$). Asterisks indicate significant differences between treatments (*$P < 0.05$, **$P < 0.01$, Student's *t*-test).

3.3 *TaCaM* gene expression and Ca^{2+} concentration

To further explore the role of Ca^{2+} signals in the wheat response to aphids, we measured Ca^{2+} concentration and *TaCaM* expression in leaves exposed and unexposed to aphids. Ca^{2+} concentration differed significantly between plants grown from $CaCl_2$-pretreated seeds and control plants over the entire experimental period. However, Ca^{2+} concentration in the leaves of plants grown from $CaCl_2$-pretreated seeds was significantly higher than in the leaves of control plants, and was 20.50%, 35.37% and 45.13% higher in the former than in controls at 24, 48 and 72 h after plant exposure to aphids (Figure 3a). By contrast, the expression levels of *TaCaM* genes showed no differences between treatments observed before plants were exposed to aphids. However, the expression of *TaCaM* genes increased sharply when plants were exposed to aphids, with levels of *TaCaM* expression 3.17-, 2.25- and 1.67-fold higher in plants grown from $CaCl_2$-pretreated seeds than in control plants at 24, 48 and 72 h after plant exposure to aphids (Figure 3b).

3.4 Callose concentration and deposition

No significant differences were observed in callose concentrations in wheat leaves of plants in the $CaCl_2$ and control treatments before plants were exposed to aphids. However, 24, 48 and 72 h after exposure, callose concentrations were 53.72%, 39.23% and 50.42% higher in the leaves of plants grown from $CaCl_2$-pretreated seeds than in control plants (Figure 4). By contrast, callose concentrations in control plants increased by only −0.03%, 23.37% and 21.95% in response to aphids. The highest callose concentration (569.32 ng g^{-1}) was found in plants grown from $CaCl_2$-pretreated seeds at 72 h after plant exposure to aphids.

After staining with aniline blue, we identified that the callose concentration was high in phloem cells of the leaf lamina. In addition, callose deposition was detected predominantly in phloem cells in vascular bundles located in the middle veins of the leaves. This result was more substantial in leaves of plants grown from $CaCl_2$-pretreated seeds than in those of control plants. At 24, 48 and 72 h after exposure to the aphids, callose deposition increased in the leaves of control plants and plants grown from $CaCl_2$-pretreated seeds. However, this result was more substantial in leaves of plants grown from $CaCl_2$-pretreated seeds than in those of control plants, as indicated by the brightness around the phloem in the image (Figure 5).

3.5 Expression of callose synthase genes in response to aphid attack

The interaction between $CaCl_2$ and aphid treatments significantly affected the expression of callose synthase (*TaGSL*) genes.

Table 2. Electrical penetration graph (EPG) variables of *Schizaphis graminum* (Rondani)

EPG parameter	Number	Variable	Treatment		*P*-value
			Control	$CaCl_2$	
General probing behavior	1	Number of probes	4.95 ± 0.59	10.05 ± 1.47	0.007**
	2	Total probing time (h)	5.70 ± 0.07	5.34 ± 0.19	0.043*
	3	Number of np	4.15 ± 0.61	9.40 ± 1.48	0.006**
	4	Total duration of np (min)	15.18 ± 2.88	36.49 ± 11.18	0.028*
	5	Mean duration of np (min)	3.47 ± 0.58	5.16 ± 1.84	0.841
Pathway phase	6	Number of C	8.00 ± 1.31	14.80 ± 1.52	0.000**
	7	Total duration of C(h)	2.06 ± 0.22	3.27 ± 0.21	0.001**
	8	Number of pd	59.45 ± 7.07	100.40 ± 11.36	0.006**
	9	Total duration of pd (min)	8.06 ± 0.75	12.67 ± 1.52	0.026*
	10	Mean duration of pd (s)	8.76 ± 0.37	7.53 ± 0.20	0.001**
Derailed stylet mechanics	11	Number of F	0.05 ± 0.05	0.80 ± 0.16	0.001**
	12	Total duration of F (min)	0.70 ± 0.70	11.20 ± 3.69	0.001**
	13	Mean duration of F (min)	0.70 ± 0.70	9.25 ± 3.36	0.001**
Xylem ingestion	14	Number of G	1.40 ± 0.84	0.35 ± 0.30	0.068
	15	Total duration of G (min)	7.01 ± 4.70	1.30 ± 1.27	0.063
Phloem phase	16	Total duration of E (min)	210.72 ± 16.40	112.38 ± 14.93	0.000**
	17	Duration of first E	61.07 ± 23.33	22.67 ± 10.18	0.038*
	18	Number of E1	3.00 ± 0.48	4.65 ± 0.47	0.004**
	19	Total duration of E1 (min)	2.91 ± 0.58	3.17 ± 0.28	0.046*
	20	Mean duration of E1 (min)	0.99 ± 0.09	0.69 ± 0.06	0.020*
	21	Number of E2	2.60 ± 0.33	4.35 ± 0.45	0.004**
	22	Total duration of E2 (min)	207.81 ± 16.49	109.21 ± 14.86	0.000**
	23	Mean duration of E2 (min)	115.56 ± 19.77	27.93 ± 5.10	0.000**
	24	Duration of longest E2 (min)	188.00 ± 18.98	69.25 ± 10.42	0.000**

Wheat plants grown from seeds pretreated with $CaCl_2$ or water were probed. Values are means ± SE ($n = 20$). Asterisks indicate significant differences between treatments (*$P < 0.05$, **$P < 0.01$, Mann–Whitney *U*-test). C, pathway phase; F, derailed stylet mechanics; G, xylem ingestion; E1, phloem salivation; E2, phloem ingestion; pd, potential drop; np, non-probe phase.

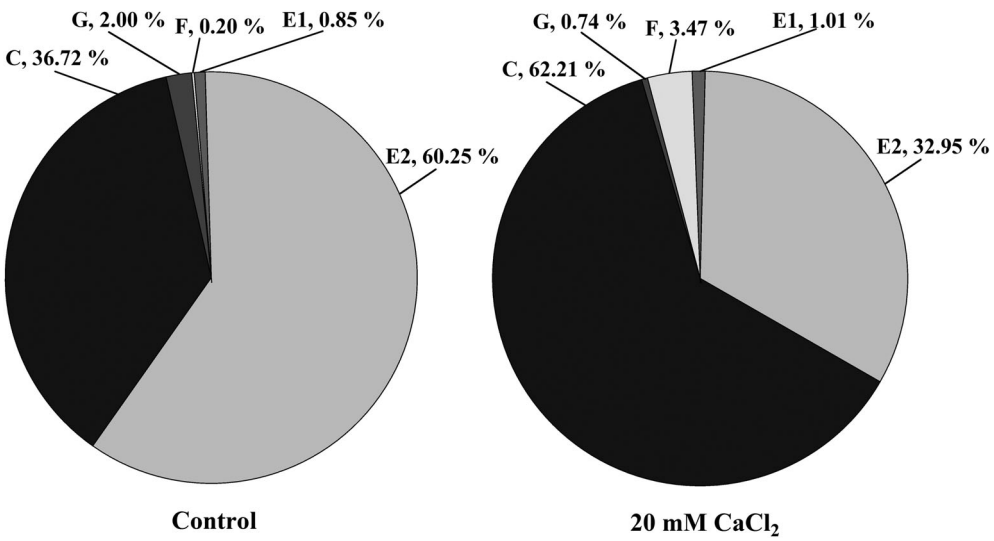

Figure 2. Percentage of total time spent in the pathway, xylem and phloem phases by wheat aphids feeding on wheat plants grown from seeds pretreated with $CaCl_2$ or water (controls). Data are means ± SE ($n = 20$). C, pathway phase; F, derailed stylet mechanics; G, xylem ingestion; E1, phloem salivation; and E2, phloem ingestion.

Specifically, the expression levels of *TaGSL2*, *TaGSL10* and *TaGSL19* were upregulated in the $CaCl_2$-pretreated seeds plant group unexposed to aphids, and markedly increased even further upon exposure to them. In addition, the expression levels of four other genes (*TaGSL8*, *TaGSL12*, *TaGSL22* and *TaGSL23*) were not significantly different between treatments but, in all cases, the increase

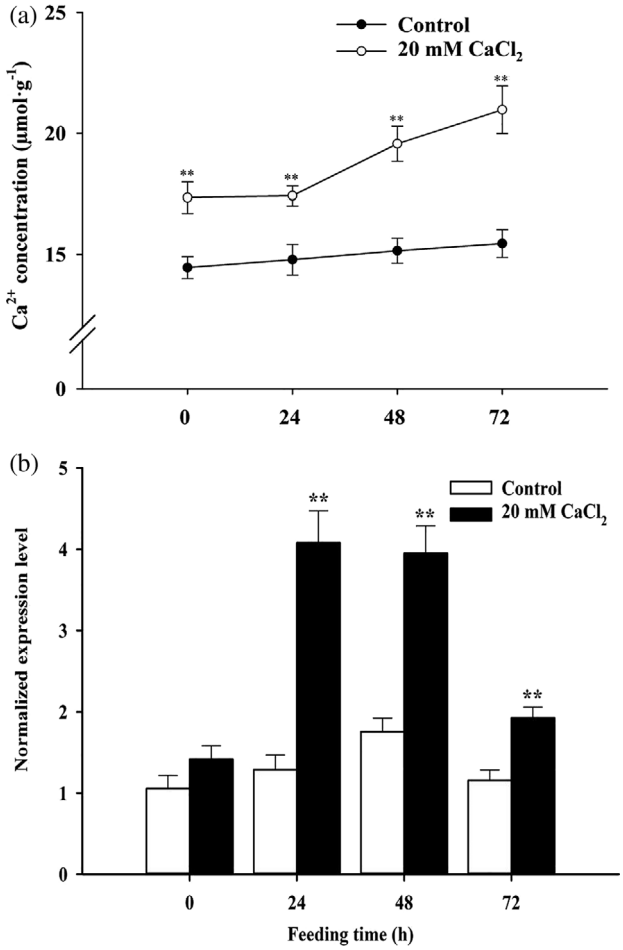

Figure 3. (a) Determination of total calcium (Ca^{2+}) concentration in wheat leaves in response to wheat aphid attack. The test was conducted 20 days after planting (three-leaf stage). Values are means ± SE (n = 10). For each time point, asterisks indicate significant differences between treatments (*P < 0.05, **P < 0.01, Student's t-test). (b) Relative expression of *TaCaM* genes in leaves exposed to aphids. Values are means ± SE (n = 3). For each time point, asterisks indicate significant differences between plants grown from seeds treated with calcium chloride ($CaCl_2$) or water (controls) (*P < 0.05, **P < 0.01, Student's t-test).

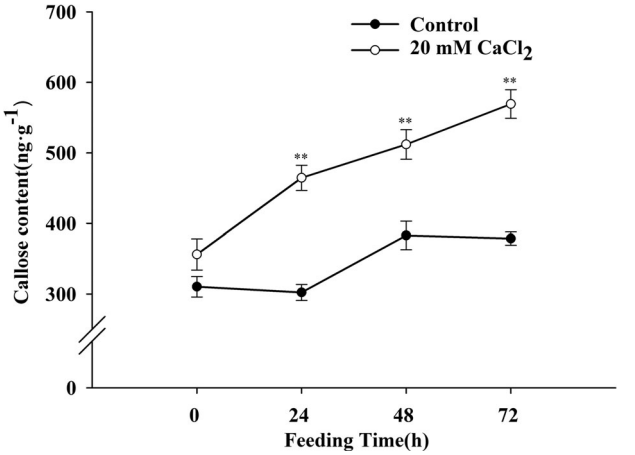

Figure 4. Callose concentration in wheat leaves in response to wheat aphid attack. Wheat seeds were soaked in 20 mM calcium chloride ($CaCl_2$) or water (controls) for 48 h. The test was conducted 20 days after planting (three-leaf stage). Values are means ± SE (n = 10). For each feeding time, asterisks indicate significant differences between treatments (*P < 0.05, **P < 0.01, Student's t-test).

in expression in response to aphid attack was higher in the $CaCl_2$-pretreated seeds plant group. The expression levels of *TaGSL8*, *TaGSL10*, *TaGSL19*, *TaGSL22* and *TaGSL23* peaked at 48 h after exposure to aphids, whereas those of *TaGSL2* and *TaGSL12* did not peak until 72 h after aphid exposure (Figure 6).

4 DISCUSSION

Seed priming results in the induction of a particular physiological state after plants are treated with natural and/or synthetic compounds before germination.[15, 60–62] Because it requires no action by growers and is economically more attractive than field application of chemicals to growing plants, several seed treatments have been developed as an effective and practical strategy to enhance plant resistance to stresses.[15]

Calcium is an essential macroelement for plant growth that has important roles in all aspects of cell function including responses to various biotic and abiotic environmental stresses.[33, 37, 63–66] In this study, pretreatment of wheat seeds with $CaCl_2$ primed plant defense mechanisms against aphid attack, which in turn reduced the aphid rate of development and population size (Table 1 and Figure 1). Consistent with our findings, Zeng *et al*. found that $CaCl_2$ significantly extended the growth period of western flower thrips.[31] These observations suggest that a resistance mechanism became active in plants grown from $CaCl_2$-pretreated seeds, which affected aphid feeding behavior and growth. EPG has been used as an effective tool to explore the feeding behavior of piercing–sucking insect pests.[49, 67] To explore the causes underlying the slower development and smaller populations of aphids on wheat plants grown from $CaCl_2$-pretreated seeds, we used EPG to evaluate aphid feeding behavior. Aphids feeding on plants grown from $CaCl_2$-pretreated seeds exhibited significantly shorter durations for total probing time compared with aphids feeding on control plants (Table 2, variable 2). In addition, the numbers of F waves and their total duration were higher in aphids feeding on plants in the $CaCl_2$-pretreatment group than in the control treatment (Table 2, variables 11 and 12). This indicates that aphid feeding induced the plant to produce more mechanical obstacle waves during probing, and that aphids feeding on plants grown from $CaCl_2$-pretreated seeds experienced greater difficulty in using the intercellular stylet pathway to reach the phloem. Xylem-feeding behavior has been demonstrated to be associated with dehydration and performance in aphids.[68] The G-wave form recorded by the EPG from aphids is correlated with the active ingestion of xylem sap,[69] frequent occurrence of xylem ingestion by dehydrated aphids indicated a need to replenish water as a result of teneral fasting.[70] Therefore, the reduction in frequency of the xylem wave form G in aphids fed on wheat plants treated with $CaCl_2$ (Table 2, variable 15) suggests a possible negative effect on the cibarial pump, which resulted in a decreased capacity to ingest xylem sap.[71] Similar results have been reported in bird cherry-oat aphid (*Rhopalosiphum padi*) reared on wheat plants treated with a sublethal dose of thiamethoxam and wheat aphid *Sitobion avenae* reared on wheat plants treated with a sublethal dose of imidaclopprid, dinotefuran, thiacloprid and thiamethoxam.[68,72] Therefore, we hypothesize that the reduction in phloem and xylem feeding had severe detrimental effects on aphids growth. In addition, aphids rely on phloem sap as their only source of nutrients.[73–75] This is particularly important because the number of E1 and E2

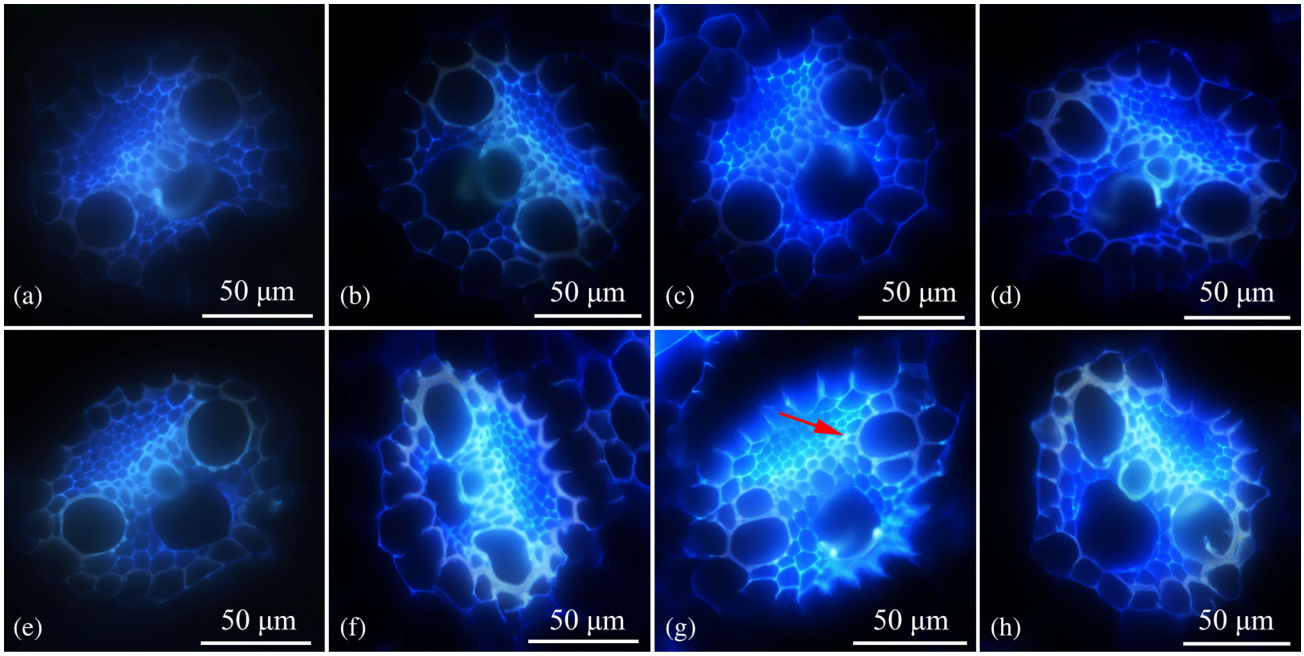

Figure 5. Callose deposition in wheat leaves in response to wheat aphid. (a) 0 h, (b) 24 h, (c) 48 h and (d) 72 h after leaves of control plants (seeds pretreated with water) were fed on by ten wingless agamic adult aphids. (e) 0 h, (f) 24 h, (j) 48 h and (h) 72 h after the leaves of plants grown from seeds pretreated with 20 mM calcium chloride ($CaCl_2$) were fed on by wheat aphids.

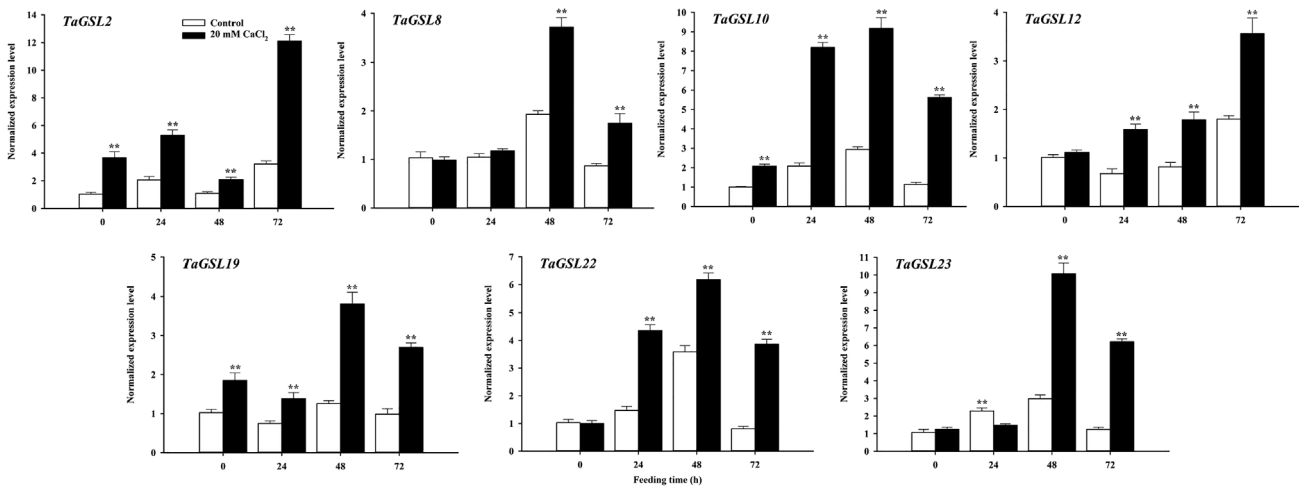

Figure 6. Relative expression of callose synthase genes (*TaGSL*) in wheat leaves fed on by wheat aphids. qRT–PCR was used to detect the level of expression of seven callose synthase genes at 0, 24, 48 and 72 h after plants were exposed to ten wingless agamic adult aphids. Values are means ± SE ($n = 3$). For each feeding time, asterisks indicate significant differences between plants grown from seeds pretreated with calcium chloride ($CaCl_2$) and plants grown from seeds pretreated with water (control plants) (*$P < 0.05$, **$P < 0.01$, Student's *t*-test).

waves was significantly higher in aphids feeding on plants grown from $CaCl_2$-pretreated seeds than in aphids feeding on control plants (Table 2, variables 18 and 21). Our EPG analysis revealed that aphids in the $CaCl_2$-pretreated seeds group needed to spend more time on penetration or on the pathway phase, experienced greater difficulty in accessing phloem sieve elements and spent more time on phloem sap ingestion. From these results (Table 2), we can infer that the phloem feeding stage was the most important difference in aphid feeding behavior between the two treatment groups in this study.

Ca^{2+} is an important messenger molecule in signal transduction, and widely participates in plant physiological and biochemical processes.[32, 36, 76] It has previously been shown that Ca^{2+} application protects plants against environmental stress, including cold injury and salt stress.[77, 78] Furthermore, Ca has previously been demonstrated to play a role in constitutive plant defenses against insect herbivory.[31] Further, previous studies have shown that the application of exogenous Ca^{2+} can significantly enhance Ca^{2+} concentration in plants,[79] which is consistent with our results. Plant immunity is a well-balanced process.[80, 81] When exposed to stress, the transient elevation of free Ca^{2+} in the cytoplast triggers a full range of signal transduction pathways via Ca^{2+}-binding proteins such as calmodulins (CaM).[82] Therefore, we measured the expression of *TaCaM* genes to examine the role of Ca^{2+} in

resisting aphid attack. When plants grown from seeds treated with $CaCl_2$ were exposed to aphids, the concentration of Ca^{2+} in the leaves increased over time, and *TaCaM* expression was induced, with gene expression levels peaking 24 h after plant exposure to aphids. In general, our results indicate that treating seeds with $CaCl_2$ resulted in plants showing higher Ca^{2+} concentrations and higher *TaCaM* expression levels in the leaves when exposed to aphids. Ca^{2+} signals are core transducers and regulators in many adaptation and developmental processes in plants.[63] Thus, Ca^{2+} accumulation in wheat leaves may be involved in resistance to wheat aphid attack.

Plants defend themselves from aphid attack through phloem-based defense mechanisms.[83–85] Callose is a natural permeable barrier and a leak-sealing compound found in plant tissues injured by herbivores.[43–46] A greater callose deposition may lead to more serious occlusion of the phloem vessels and cause greater difficulty for aphids during prolonged feeding.[45, 86] Callose deposition is one of the direct defense responses of host plants to stop insect feeding. Additionally, growing evidence indicates that glucan synthase-like (*GSL*) genes are involved in callose synthesis in higher plants.[54] We analyzed the expression levels of seven *GSL* genes (*TaGSL*), as well as callose deposition and total callose concentration in the leaves. No clear differences were observed between treatment groups regarding callose concentrations or deposition in the leaves when plants were not exposed to aphids. However, increased callose deposition in the leaves of plants grown from $CaCl_2$-pretreated seeds were observed after exposure to aphids (Figure 4). Measurements of the expression levels of callose synthase genes (*TaGSL2*, *TaGSL10* and *TaGSL12*) support the theory that treating seeds with $CaCl_2$ primes future plant defenses.[54–56] Exogenous Ca only slightly increased leaf callose concentration in the absence of aphids, whereas callose increased sharply, together with the expression levels of *TaGSL8*, *TaGSL19*, *TaGSL22* and *TaGSL23*, when plants in the $CaCl_2$-pretreatment group were exposed to aphid attack (Figure 6). Aniline blue staining of plant tissue sections revealed that callose depositions were mainly located in the phloem (Figure 5). In addition, the main role of Ca^{2+} in plant–aphid interactions is believed to occur in the phloem because in the phloem Ca^{2+} promotes occlusion via regulation of callose production. Altogether, our results provide a comprehensive view of callose regulation, callose synthesis-related gene expression and localization, and Ca^{2+} concentration in wheat plants in response to aphid attack.

Ca^{2+} signals are crucial for the activation of plant immune responses.[39, 87–90] For example, *CPK5* of the plant calcium-dependent protein kinase family in *Arabidopsis thaliana* was identified as a positive regulator of innate immune signaling, with a dual function in rapid signal propagation and the induction of prolonged transcriptional and salicylic acid-mediated defense responses facilitating plant resistance to bacterial pathogens.[91] In addition, recent evidence suggests that calcium signals are also involved in nucleotide-binding and leucine-rich repeat-mediated plant immune responses.[92, 93] Here, our results showed that seed priming with $CaCl_2$ leads to stronger defensive responses to aphid infestation, including higher Ca contents and expression of *TaCaM* compared with control plants, suggesting that Ca^{2+} signals are involved in immune responses induced by exogenous $CaCl_2$. Although the involvement of Ca in wheat resistance against aphids has been demonstrated here, the underlying detailed mechanism remains largely obscure. Many studies have reported that changes in chromatin structure, DNA methylation and histone modification are responsible for priming of plants against abiotic and biotic stresses in plants.[94–100] We thus propose that the $CaCl_2$-mediated priming against aphids we observed here may also involve epigenetic modifications of Ca-responsive genes in embryonic tissues during seed imbibition.

In conclusion, seed treatment with calcium chloride enhances wheat resistance against wheat aphid, a destructive insect pest of wheat. The Ca^{2+}-increased resistance is closely associated with callose deposition. This study provides a desired approach to increase plant resistance and control wheat aphid by treating seeds with exogenous calcium, which likely reduces pesticide application.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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