Immunogold labelling of TIP proteins in the resurrection plant *Craterostigma plantagineum.*

By
Victoria Maguire

Supervisor
Dr Jill Farrant

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Faculty of Science,
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Abstract

The aim of this project was to study whether the concentrations of Tonoplast Intrinsic Proteins (TIPs) change during drying in *Craterostigma plantagineum* in order to determine whether TIPs play a part in the replacement of vacuolar contents and desiccation tolerance in general. Vacuoles of resurrection plants are filled with non-aqueous substances during drying. The replacement of the water in their vacuoles prevents mechanical damage that would occur with a loss of turgor (Farrant, 1999).

Leaf tissue from *Craterostigma plantagineum* of relative water content (RWC) 100%, 50% and 25% were fixed and embedded in Spurr's resin. The tissues were sectioned and immunogold labelled using α-, γ- and δ- TIP antibodies. Sections viewed with the transmission electron microscope showed unspecific labelling in the cell wall, chloroplast and cytoplasm. In order to determine what was causing the unspecific labelling, various optimisation procedures were tried. These included: - (1) using different primary antibody (TIP) concentrations, (2) different secondary antibody (anti-rabbit gold label) concentrations, (3) use of Spurr's resin vs. LR White resin, and (4) fixation with and without osmium. For all these treatments there was still unspecific labelling, except for the grids where very low concentrations of primary antibody were used where no gold particles could be seen.

A Western blot analysis using αTIP antibody and showed background binding of the antibody to the cellulose material used. The conclusion reached was that the cause of the

unspecific labelling was a combination of an insufficiently specific antibody, which labelled cellulose and some general unspecific labelling by the secondary gold label.

Although the original aim of the project was not addressed, this work allowed evaluation of protocols for immunogold labelling with TIP antibodies. This could be used as a basis for further studies into this subject.

Introduction

Membrane intrinsic proteins (MIPs) are a highly conserved group of proteins that appear to be ubiquitous throughout the animal and plant kingdoms. However plants seem to contain many more MIPs than any other type of organism (Barkla *et al*, 1999). The main reason for the great number of MIPs in plant cells is their role in water movements and the control of water flux (Barkla *et al*, 1999; Tyerman *et al*, 1999). The compartmentation of the vacuoles of plant cells requires a control of water and solute transport across both the tonoplast and plasma membrane (Tyerman *et al*, 1999). There are typically three families of MIPs recognised; the tonoplast intrinsic proteins (TIPs), the plasma membrane intrinsic proteins (PIPs) and nodulin-26 (expressed in the peribacteroid membrane of root symbiotic nodules) (Maurel, 1997; Barkla, 1999; Chrispeels & Maurel, 1994).

Aquaporins are a particular type of MIP that support water flow through membranes. The general role of aquaporins is to regulate transmembrane water transport while the plant is growing, developing and during a stress response (Maurel, 1997). Some TIPs function as aquaporins but other TIPs have been shown to transport proteins across the vacuole membrane.

Aquaporins have a molecular mass of between 26 and 30kDa (Tyerman *et al*, 1999). Their structure consists of six membrane spanning α helices with the N and C termini both located on the cytoplasmic side of the membrane (Tyerman *et al*, 1999; Chrispeels

& Maurel, 1994; Knepper, 1994; Maurel, 1997). The two halves of the polypeptide are obversely symmetrical and the loops containing the NPA motif overlap in the middle of the lipid bilayer. This overlap forms two hemipores, which form a narrow, water-filled channel (Tyerman *et al*, 1999; Chrispeels & Maurel, 1994; Knepper, 1994; Maurel, 1997). It has been suggested that the structure of the aquaporins accounts for their ability to mediate bi-directional water flow (Tyerman *et al*, 1999). Water flows passively through the narrow pores of aquaporins because of a free energy gradient (Tyerman *et al*, 1999; Chrispeels & Maurel, 1994). Some aquaporins, such as γTIP and αTIP, are highly selective for water while others also allow non-electrolytes to travel through them as well (Tyerman *et al*, 1999; Chrispeels & Maurel, 1994).

Various hormonal and environmental factors can affect the expression of aquaporins. By differentially screening cDNA libraries γTIP and RD28 have been identified as genes that are upregulated by gibberellic acid and drying treatments respectively (Maurel, 1997). Aquaporins that are regulated by water stress could contribute to the drought tolerance of plants. It is not clear exactly what part aquaporins would play in helping plants cope with water deficit, they could be involved in adjusting the overall hydraulic conductivity of the plant, moving water towards critical cells and organs or helping preadapt desiccated tissue to rehydration stresses (Maurel, 1997). It would seem that aquaporins are definitely involved in plant cell turgor and volume regulation. This would be especially important during seed germination where desiccated cells must rehydrate and after the plant has been exposed to drought stresses (Maurel, 1997).

Johnson *et al* (1989) isolated an abundant, highly conserved TIP (TP25) from *Phaseolus vulgaris* seeds. They studied the protein's intracellular and tissue distribution and concluded that TP25 played a role in maintaining the tonoplast during dehydration or rehydration of the seed. This TIP is highly conserved among different species and it is only found in seeds (Johnson *et al*, 1989).

Relatively few immunocytochemical studies have been performed on TIPs in the vegetative tissues of plants (Barkla *et al*, 1999). Immunofluorescence studies done by Jauh *et al* (1998; 1999) have shown that TIPs can be used as markers for vacuolar functions. Seed-type protein storage vacuoles are marked by the presence of αTIP, lytic vacuoles are marked by the presence of γTIP and vegetative protein and pigment storage vacuoles are marked by the presence of δTIP, indicating that specific TIP isoforms correlate with the function of the vacuole (Jauh *et al*, 1998).

The aim of this project was to determine the subcellular location of the three TIPs in the resurrection plant *Craterostigma plantagineum*. The TIPs were labelled using standard immunogold labelling techniques, using antibodies to α-, γ- and δ- TIP as well as colloidal gold as a marker, and viewed using a transmission electron microscope (TEM) to determine whether there is a change in the numbers of these TIPs found in the tonoplast at different stages of drying.

Resurrection plants are a group of angiosperms that are desiccation tolerant, meaning that they can revive from an air-dried state (Farrant *et al*, 1999). *Craterostigma plantagineum* (Scrophulariaceae) is the most extensively studied resurrection plant. It can survive drying of it's vegetative tissue to 15% of fresh weight, over a period of between 24 and 48 hours (Oliver & Bewley, 1997). The leaves of *Craterostigma plantagineum* shrink when dehydrated, with the area decreasing to less than 15% of the area when fully turgid when the water content is approximately 5% (Hartung *et al*, 1998).

Resurrection plants have a combination of strategies to protect their tissues during drying and rehydration (Bray, 1997; Farrant *et al*, 1999; Farrant, 2000; Hartung *et al*, 1998; Oliver & Bewley, 1997). These protection strategies can be mechanical in nature. For instance *Craterostigma* spp. have extensive wall folding that reduces the stress on the cell membrane during drying (Farrant, 1999; Hartung *et al*, 1998) and *Xerophyta* spp. replace the water in their vacuoles with compatible solutes to prevent mechanical damage that would occur with a loss of turgor (Farrant, 1999). In the resurrection plants *Craterostigma wilmsii*, *Myrothamnus flabellifolius* and *Xerophyta humilis* the large water filled vacuoles present in the fully hydrated plants are replaced by smaller vacuoles that are filled with non-aqueous substances during drying (Farrant, 2000). The degree of vacuolation varies from species to species, as do the other protection mechanisms that come into play during water stress (Farrant, 2000).

The nature of the substances that fill the vacuoles during drying is not known (Farrant, 2000). The plant must have some mechanism of removing the water from the vacuoles and transporting new substances into the vacuole. If there is an increase in TIPs found on the tonoplast during drying it could indicate that TIPs are being used to move water out of the vacuole and bring other substances in to replace it. The aim of this project is to do a preliminary study of whether the concentrations of TIPs change during drying in *Craterostigma plantagineum* in order to determine whether TIPs play a part in the replacement of vacuolar contents and desiccation tolerance in general.

Methods

Leaf material was obtained from plants of *Craterostigma plantagineum* at different stages of drying. The plants had been maintained in the University of Cape Town greenhouse under ambient environmental conditions before the start of the experiment and were healthy and well watered. The plants were placed in the phytotron at 60% humidity, 26°C (day temperature) and a cycle of 14 hours of light and 10 hours of darkness for the duration of the experiment. The plants were allowed to acclimatise to phytotron conditions for several days prior to commencement of drying.

Approximate drying times were obtained from the paper by Farrant *et al* (1999), with modifications to account for differences in temperatures and humidity of the phytotron environment. The plants were watered prior to the start of experimentation. Water was then withheld for the duration of the experiment (6 days). One leaf was taken from a plant on day 1, day 4 and day 5 to obtain estimated water contents of 100%, 50% and 25% respectively.

For each water content half of the leaf was used for calculation of relative water content and the other half of the leaf was prepared for microscopical studies. Leaf water content was determined gravimetrically by oven drying at 70°C for 48 hours. Water content and relative water content (RWC) were determined using the equations below.

$$\text{Water content (WC)} = \frac{\text{fresh mass} - \text{dry mass}}{\text{dry mass}}$$

$$\text{Relative water content (RWC)} = \frac{WC_{\text{sample}}}{WC_{\text{at full turgor}}} \times 100\%$$

Leaf material was trimmed into 5mm by 5mm squares and these were fixed in a 2.5% glutaraldehyde and 0.5% caffeine solution in 0.1M phosphate buffer, pH 7.2, overnight. The leaf material was then washed three times for five minutes each in 0.1M phosphate buffer (pH = 7.2). Half the material was post fixed in 1% osmium tetraoxide in 0.2M phosphate buffer and the remainder was kept in buffer only. After one hour the material was washed again (three times for five minutes each).

The leaf material (both those fixed by osmium and those not) was then all put through a dehydration sequence. The material was immersed for two five-minute periods in a series of alcohol solutions, first 30%, then 50%, 70%, 90% and 95%. The material was then transferred to 100% alcohol for a further two ten-minute periods. The final step of the dehydration sequence was to place the material into acetone for 20 minutes (two periods of 10 minutes each).

After the material was dehydrated it was placed in one part acetone and one part thawed Spurr's (Spurr, 1969) resin (i.e. an 50% resin solution). After 4 hours half the solution was removed and replaced with pure resin to make a 75% solution. This process was repeated after another 4 hours to make an 87.5% solution. After a further 4 hours the samples were placed in pure resin and left for another 4 hours. After they had been impregnated by resin the material was placed into resin filled block moulds. These moulds were placed in the oven for 16 hours at 60°C to harden.

Tissue was sectioned using a microtome (Reichart Ultracut S) and was collected onto nickel grids. Immuno gold labelling was then done on these grids using the three TIP antibodies (α-, γ-, δ- TIP) that were donated by Dr J. Rogers (Washington State University). Antibodies were initially in a freeze-dried state and had to be reconstituted. For each antibody various dilutions were made using 0.1M Phosphate buffer (PBST) (Table 1).

Grids were labelled using the following procedure. Grids were incubated on 5µl drops of 1% bovine serum albumen (BSA) for 30 minutes. They were then placed on 5µl drops of the primary antibody (either α-, γ- or δ- TIP diluted to the appropriate concentration) and left overnight in the fridge on damp filter paper. Each grid was then washed in 40 drops of 0.5M PBST buffer before being placed on a 5µl drop of the secondary antibody (anti-rabbit) for one hour. Each grid was washed in 20 drops of 0.5M PBST buffer and 20 drops of distilled water.

Grids were stained before viewing using the Transmission Electron Microscope (TEM Zeiss 109). Equal amounts of 2% urynal acetate and lead citrate were centrifuged in separate eppendorfs for 3 minutes prior to staining. Grids were placed on 5µl drops of urynal acetate for 10 minutes and then washed in 40 drops of water. They were then placed on 5µl drops of lead citrate for 10 minutes in a covered Petri dish with concentrated NaOH in it to remove excess carbon dioxide that would cause a precipitate on the grids. The grids were then thoroughly washed in a flow of distilled water. Grids were viewed using the TEM.

Sections of *Craterostigma plantagineum* embedded in LR White resin were kindly donated by M. Vicre (Dept Biochemistry, UCT). The labelling and staining procedures were the same for these grids except that LR White grids were left on the lead citrate for only 60 seconds. A Western blot analyses on αTIP was performed by Clare van der Willigen (Dept Biochemistry, UCT).

The following treatments were viewed: - (1) tissues at different relative water contents, (2) tissues with and without osmium, (3) tissues embedded in Spurr's resin and LR White resin, (4) tissues labelled with each of the three TIPs, and (5) with different concentrations of both primary and secondary antibodies. Control grids were also viewed. Controls were treated exactly the same as the other grids except that Control 1 was placed on buffer instead of primary antibody and control 2 was placed on buffer instead of the secondary antibody. Table 1 shows the variables for each grid that was tested.

Table 1: - Variables for each grid labelled for viewing.

Approx RWC	**Osmium/ no osmium**	**Resin**	**TIP**	**Conc. Primary antibody**	**Conc. secondary antibody**	
100%	Osmium	Spurr's	α	Pure	1 in 20	
100%	Osmium	Spurr's	δ	Pure	1 in 50	
100%	Osmium	Spurr's	δ	1 in 2	1 in 50	
100%	Osmium	LR white	δ	Pure	1 in 50	
100%	Osmium	LR white	δ	1 in 2	1 in 50	
100%	Osmium	Spurr's	δ	1 in 2	1 in 50	
100%	Osmium	Spurr's	δ	1 in 2	1 in 50	
50%	No osmium	Spurr's	α	Pure	1 in 20	
50%	Osmium	Spurr's	α	Pure	1 in 20	
50%	Osmium	Spurr's	γ	1 in 5	1 in 20	
50%	Osmium	Spurr's	γ	1 in 10	1 in 20	
50%	Osmium	Spurr's	γ	Pure	1 in 50	
50%	Osmium	Spurr's	γ	1 in 2	1 in 50	
50%	No osmium	Spurr's	γ	Pure	1 in 50	
50%	No osmium	Spurr's	γ	1 in 2	1 in 50	
50%	Osmium	LR White	γ	Pure	1 in 50	
50%	Osmium	LR White	γ	1 in 2	1 in 50	
25%	Osmium	Spurr's	δ	1 in 2	1 in 50	
25%	Osmium	Spurr's	δ	1 in 2	1 in 50	
100%	Osmium	Spurr's	δ	0	1 in 50	} Controls
100%	Osmium	Spurr's	δ	1 in 2	0	} Controls

Photographs were taken showing various different aspects of the grids viewed. The negatives of these were scanned using a Nikon film scanner (LS4500AF). The position and degree of specificity of gold particles was noted for each treatment.

Results and discussion

Unfortunately due to technical difficulties (described below) during the course of the project I was unable to address the initial aim of this project: whether there is a change in the number of tonoplast intrinsic proteins (TIPs) at different stages of drying in *Craterostigma plantagineum*. However the process that I went through to identify what the problem was is useful as a diagnostic tool for future studies. In this section I will outline the results that I obtained at each stage of the experiment and suggest some ways of overcoming the problems encountered.

Initially, tissues from fully hydrated leaves (100% RWC) of *Craterostigma plantagineum* were labelled using pure αTIP antibody and an anti-rabbit gold concentration of 1 in 20. In order for the labelling to be specific it would have been exclusively located in the vacuole membrane or other membranes that might have included similar proteins. Gold particles could be seen in the cell wall, chloroplasts as well as the vacuoles leading to the conclusion that the labelling was unspecific (Fig. 1). Tissues with a RWC of 50% were also labelled with pure αTIP antibody and a gold concentration of 1 in 20. These grids also showed extensive unspecific labelling of the sections (Figs. 2 and 3).

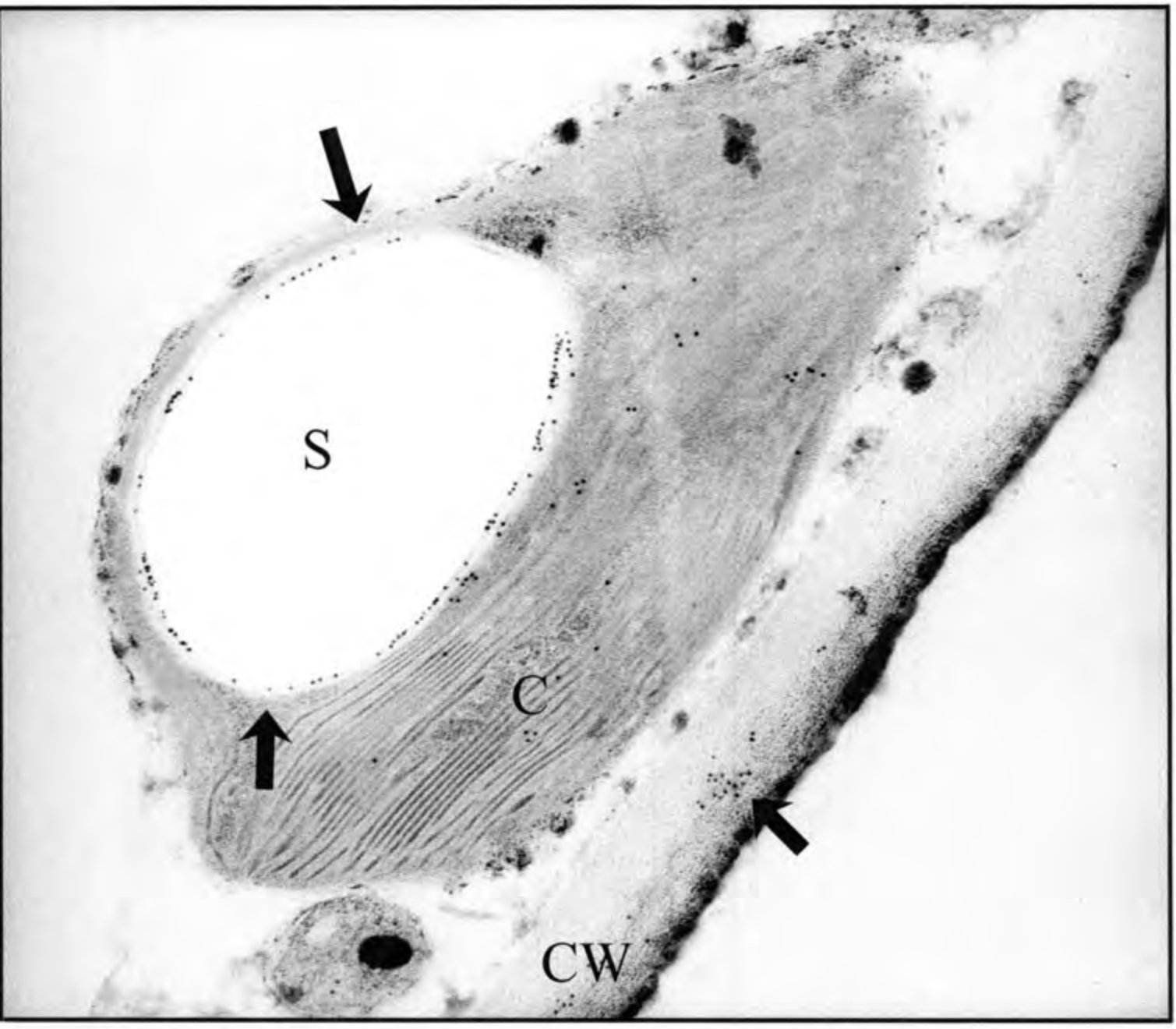

Figure 1: - Transmission electron micrograph showing the chloroplast (C), cell wall (CW) and a starch grain (S) with unspecific gold particles in leaf tissue with a RWC of 100%. Arrows point to the gold labelling. antibody conc.=pure, gold conc.=1:20. (x30000)

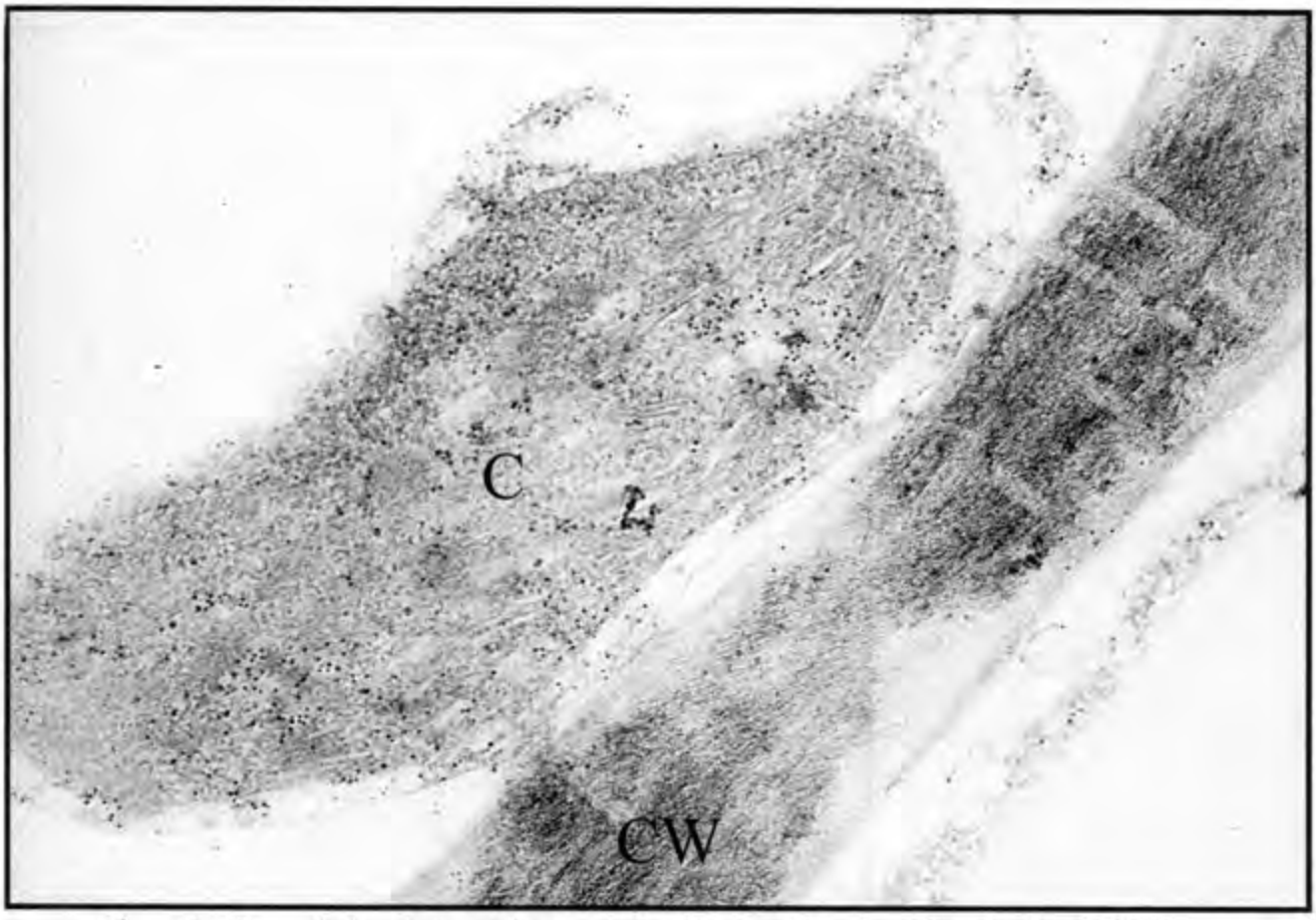

Figure 2: - Transmission electron micrograph showing the chloroplast (C) and cell wall (CW) with unspecific gold particles (black dots all over section) in leaf tissue with a RWC of 50% antibody conc.=pure, gold conc.=1:20. (x20000)

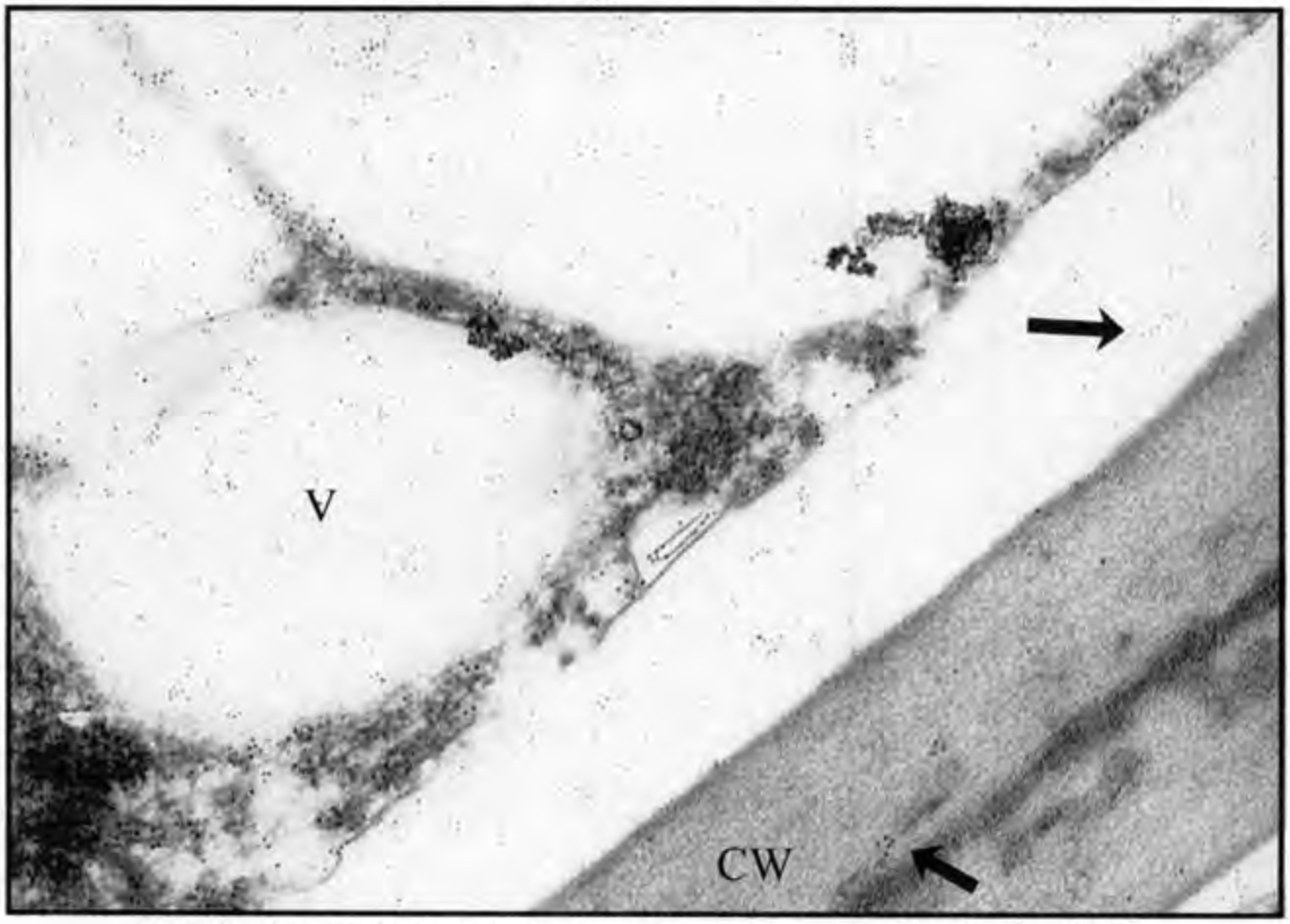

Figure 3: - Transmission electron micrograph showing the vacuole (V) and cell wall (CW) with unspecific gold particles in leaf tissue with a RWC of 50%. Arrows point to some of the gold labelling. antibody conc.=pure, gold conc.=1:20. (x20000)

In order to test whether the concentrations of the antibody were too high, and whether that was the cause of the unspecific binding to non-target areas, lower concentrations of the primary antibodies were used. Using tissues with a RWC of 50% and the γ TIP antibody, grids were prepared with the antibody at concentrations of 1 in 5 and 1 in 10. The gold concentration for both sets of grids was 1 in 20. The grids with a primary antibody concentration of 1 in 5 had some gold particles but they were not specific and were present in the cell wall and chloroplasts (Figs. 4 and 5). No gold particles could be seen on the grids with the primary antibody concentration of 1 in 10 (Fig. 6).

Another possible explanation for the unspecific labelling was that the concentration of the gold used as the secondary antibody was too high. If the concentration of gold were too strong it would have a greater tendency to label unspecifically. For the remainder of the grids used in this experiment the gold concentration was lowered to 1 in 50. Leaf tissue with a RWC of 50% was used with pure and 1 in 2 γ TIP antibodies. The grids using the pure antibody showed unspecific labelling, although there were not as many gold particles as were present in the sections using gold at 1 in 20. Unspecific labelling was noted in the tonoplast (Fig. 7) as well as the cell wall and chloroplasts (Fig. 8). The grids using the antibody at a concentration of 1 in 2 had fewer gold particles. There were no particles in the chloroplasts but there were particles in the vacuoles, however these particles were not specific and were not near the membranes.

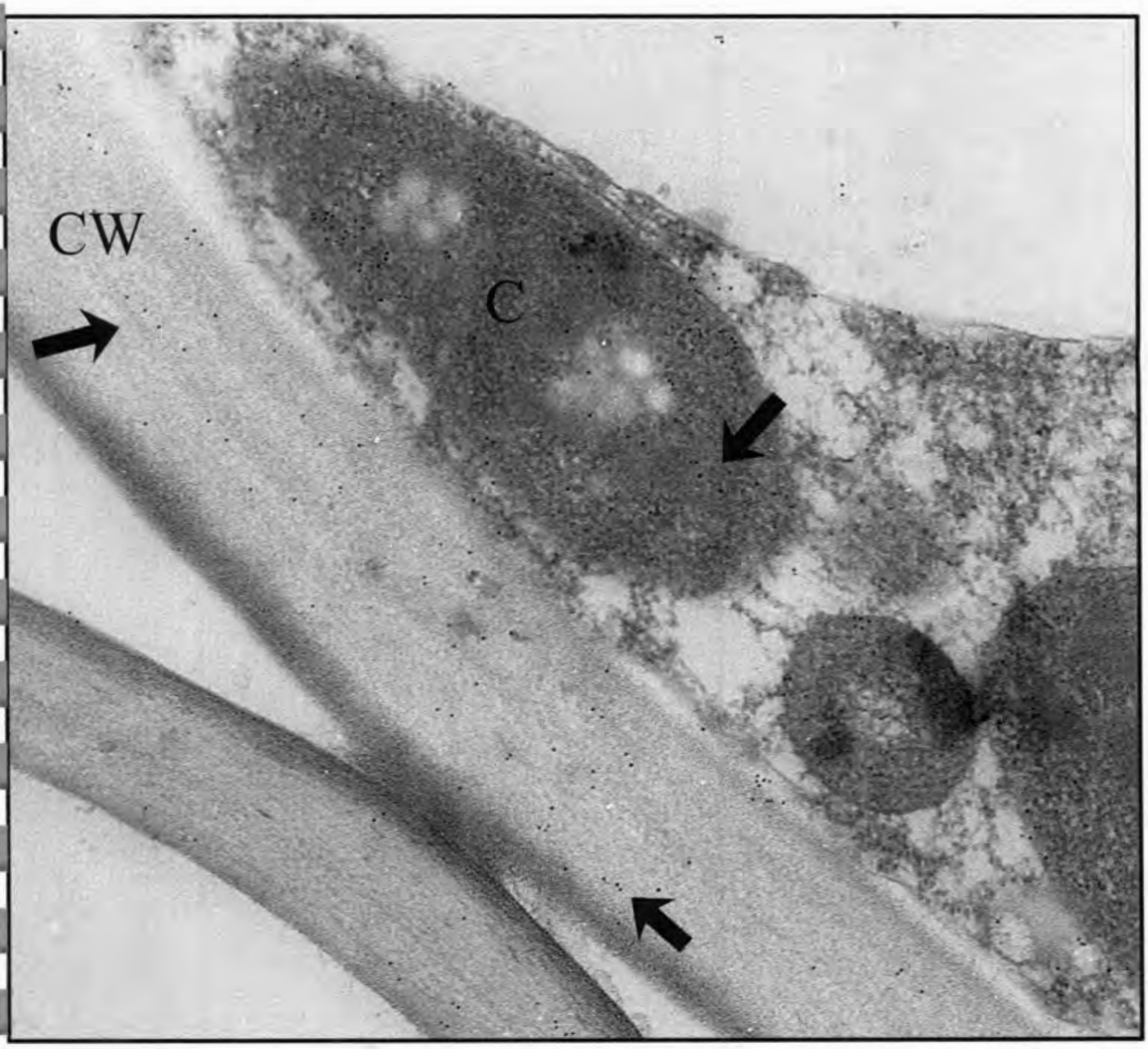

Figure 4: - Transmission electron micrograph showing the chloroplast (C) and cell wall (CW) with unspecific gold particles in leaf tissue with a RWC of 50%. Arrows point to some of the gold labelling. antibody conc.=1:5, gold conc.=1:20. (x20000)

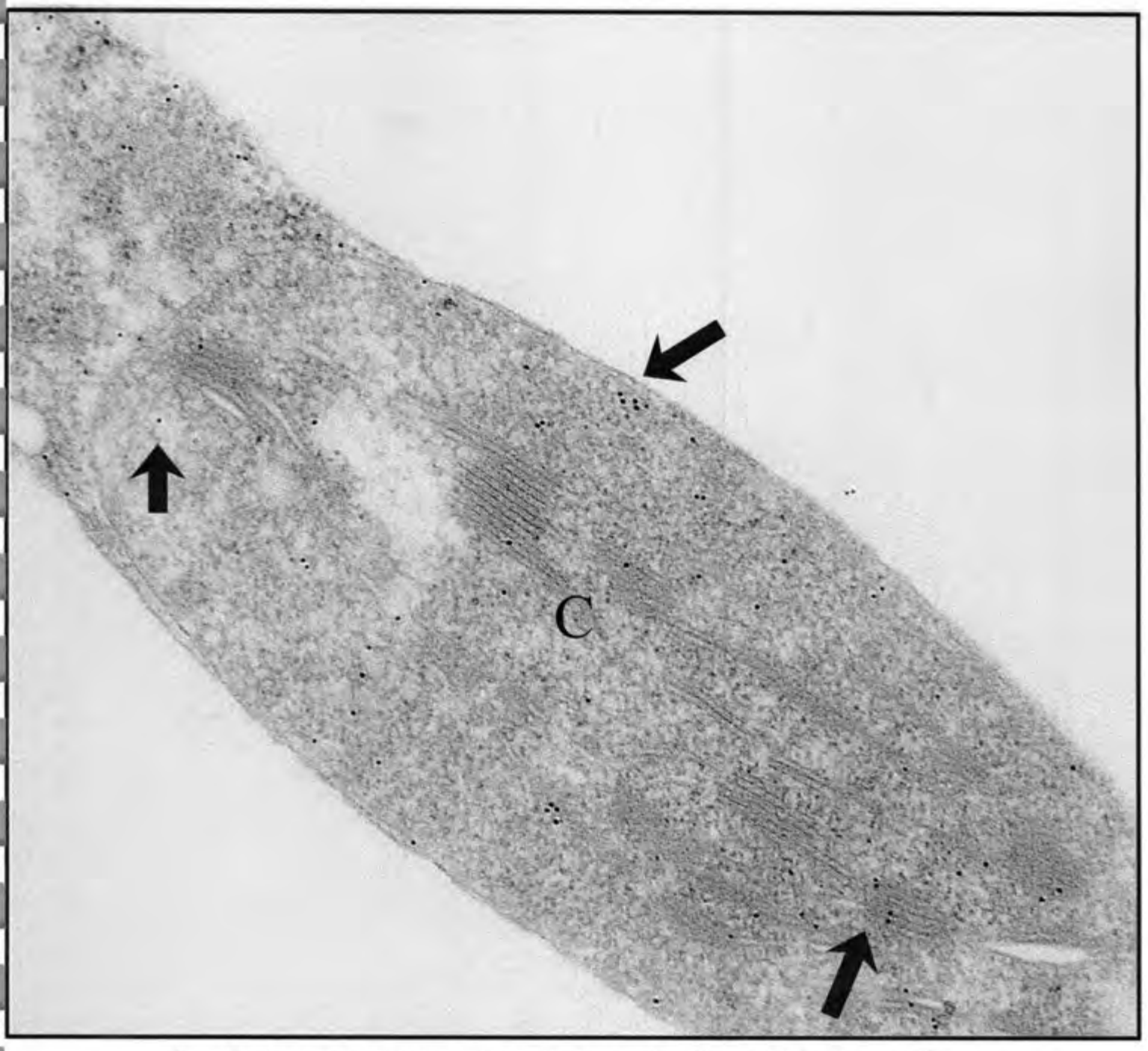

Figure 5: - Transmission electron micrograph showing the chloroplast (C) with unspecific gold particles in leaf tissue with a RWC of 50%. Arrows point to some of the gold labelling. antibody conc.=1:5, gold conc.=1:20. (x30000)

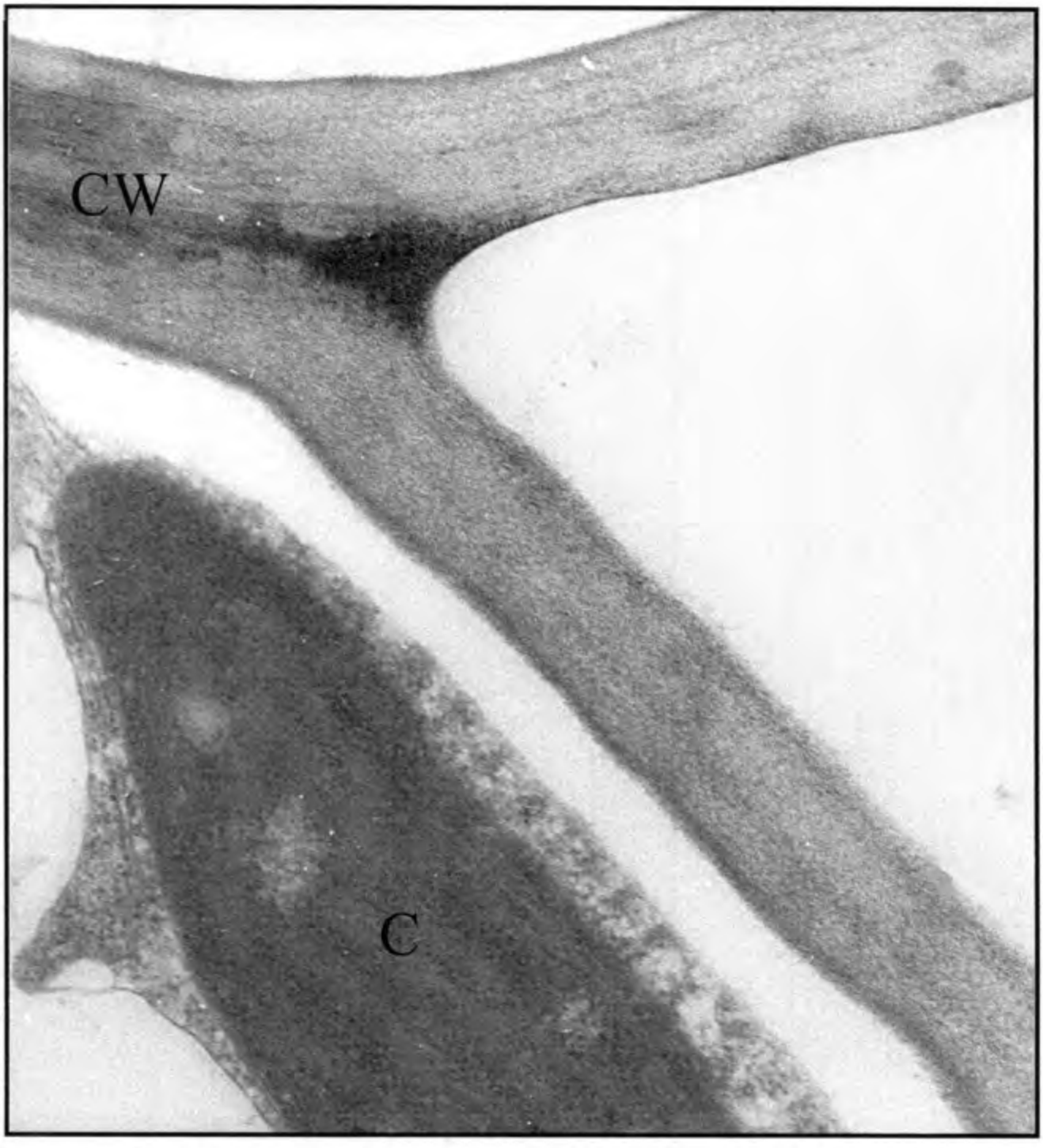

Figure 6: - Transmission electron micrograph showing the chloroplast (C) and cell wall (CW) with no gold particles in leaf tissue with a RWC of 50%. antibody conc.=1:10, gold conc.=1:20 (x30000)

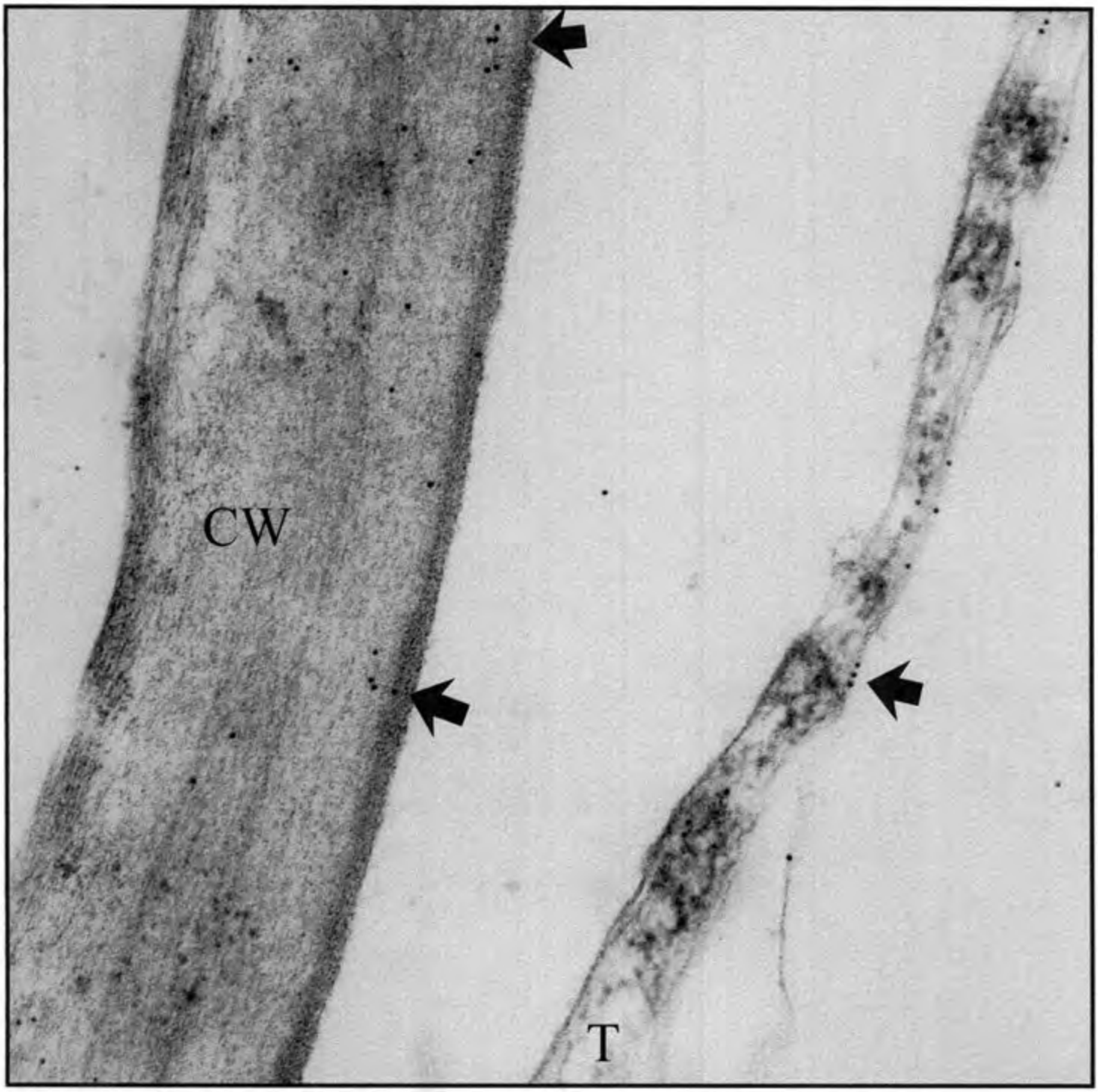

Figure 7: - Transmission electron micrograph showing the tonoplast (T) and cell wall (CW) with unspecific gold particles in leaf tissue with a RWC of 50%. Arrows point to the gold labelling. antibody conc.= 1:2, gold conc.=1:50. (x30000)

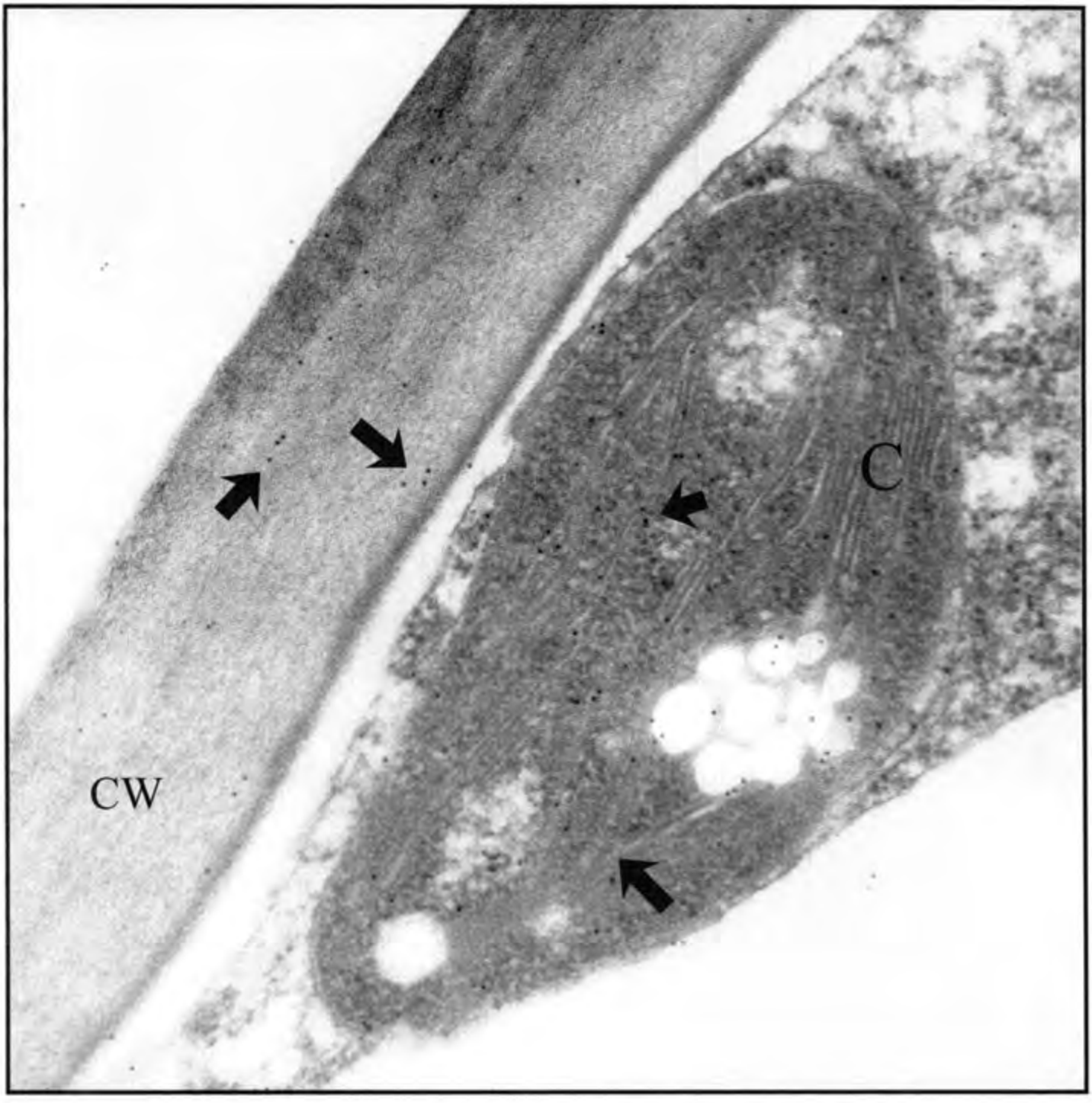

Figure 8: - Transmission electron micrograph showing unspecific gold particles in the cell wall (CW) and chloroplast (C) of leaf tissue with a 50% RWC. Arrows point to gold particles. antibody conc.=1:2, gold conc.=1:50. (x20000)

It has been suggested that use of osmium tetraoxide in the fixation of samples before embedding can be responsible for the non-labelling of proteins in sections (Varndell & Polak, 1987). Osmium is a fixative and may cause the proteins to become fixed such that the gold particles do not attach to them, effectively masking the protein antigen. Unspecific labelling elsewhere on the sections could be explained by insufficient specificity of the antibody. Sections where osmium had been used in the embedding procedure were compared to sections where osmium was not used. The major disadvantage of using samples that are not fixed by osmium is that the preservation of the material is usually poor, compared to samples where osmium is used as a fixative (Varndell & Polak, 1987). Grids of 50% RWC *Craterostigma plantagineum* that had not been exposed to osmium were labelled using pure γ TIP antibody and a gold concentration of 1 in 50. Fewer gold particles were evident on these sections (Figs 9 and 10). This could be because of bad fixation that produced inferior sections. Some gold particles could be seen near the cell wall, the tonoplast and the chloroplast but they were not specific (Figs. 9 and 10).

Unspecific labelling was still evident in the sections without osmium. This indicated that the use of osmium was not the cause of the unspecific labelling. Another explanation was that the Spurr's resin used to embed the samples was covering the active sites of the proteins and causing the lack of specific labelling on the tonoplast (Varndell & Polak, 1987). Again, unspecific labelling elsewhere on the sections could be explained by insufficient specificity of the antibody. Epoxy resins such as Spurr's can affect the antigenicity of a sample while acrylic resins such as LR White have not shown this

phenomenon (Varndell & Polak, 1987). Some sections of *Craterostigma plantagineum* embedded in LR White resin were kindly made available to me by Maite Vicre. These sections in LR White were compared to the sections that were embedded in Spurr's resin. When Leaf tissue at 50% RWC embedded in LR white was labelled using the pure γ TIP antibody and a gold concentration of 1 in 50 unspecific labelling was observed (not shown).

A direct comparison was made between 100% RWC leaf tissue embedded in Spurr's resin and LR White resin. Grids were labelled with pure δ TIP antibody and a gold concentration of 1 in 50. The sections embedded in Spurr's resin showed unspecific labelling with particles near the tonoplast but also in the cell wall (Fig. 11) and chloroplast. The interior of the vacuoles appeared to be clear of gold particles. The LR White embedded sections also showed unspecific labelling. Particles could be seen in the cell wall, chloroplast (Fig. 12), tonoplast and cytoplasm.

Unspecific labelling was evident in both the samples embedded in Spurr's and LR White. This indicates that it is not the choice of resin that is causing the unspecific labelling. I concluded that it was the specificity of the antibody used that should be questioned. If the antibody were not specific enough to the proteins that it should be targeting then this would cause the unspecific labelling that I had observed. The lack of labelling in tonoplast membranes could be due to, 1) no proteins present, 2) inappropriate antibody dilution, or 3) masking of active site of antigen (reasons described previously).

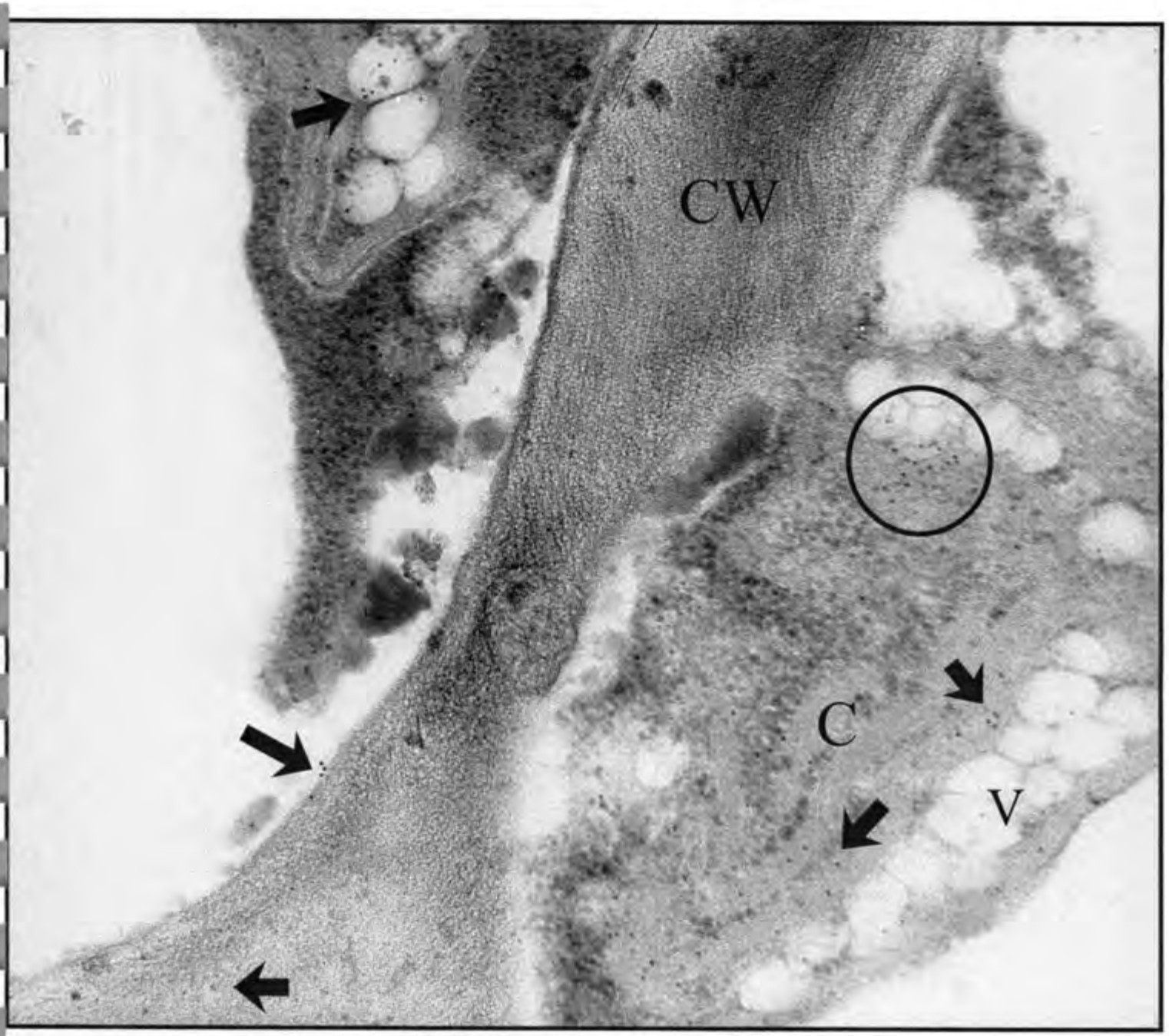

Figure 9: - Transmission electron micrograph showing unspecific labelling in the cell wall (CW), vacuoles (V) and chloroplast (C) of leaf tissue at 50% RWC where osmium was not used as a fixative during embedding. Arrows and circle show gold particles. antibody conc.=pure, gold conc.=1:50. (x30000)

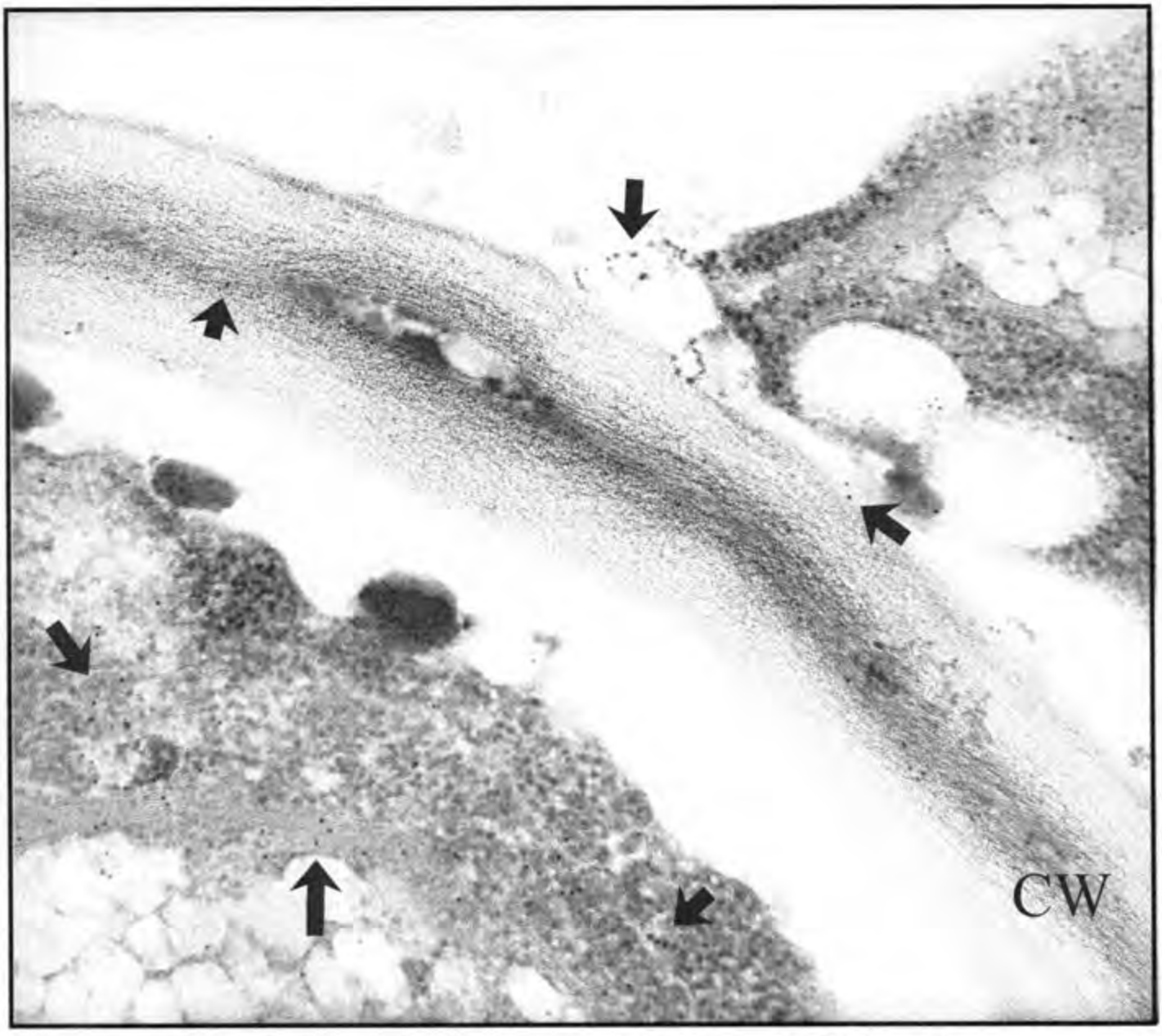

Figure 10: - Transmission electron micrograph showing unspecific labelling in the cell wall (CW) and cytoplasm of leaf tissue at 50% RWC where osmium was not used as a fixative during embedding. antibody conc.=pure, gold conc.= 1:50. (X30000)

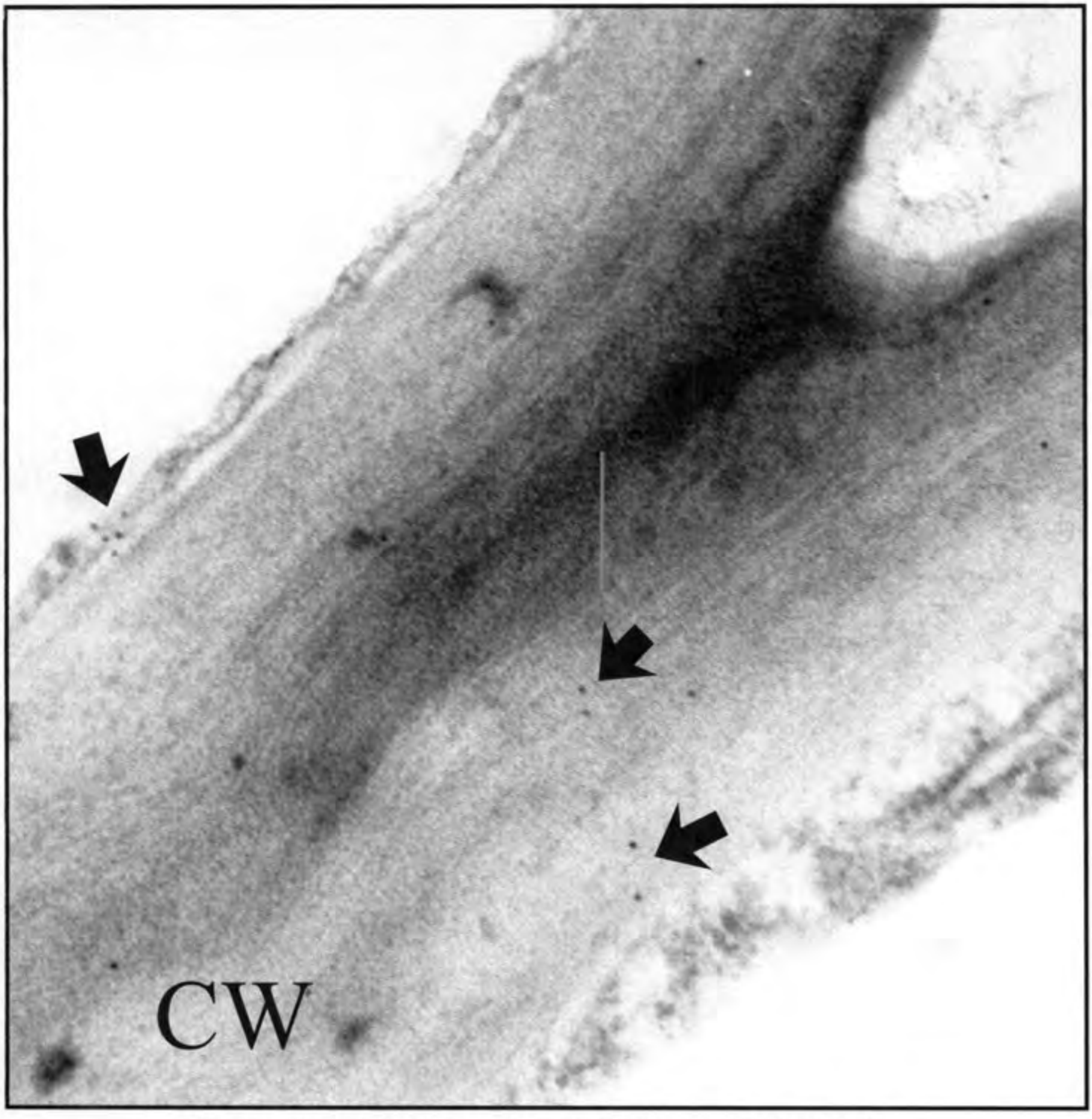

Figure 11: - Transmission electron micrograph showing unspecific labelling in the cell wall (CW) of leaf tissue at 100% RWC embedded in Spurr's resin. Arrows point to gold particles.antibody conc.=pure, gold conc.=1:50. (x30000)

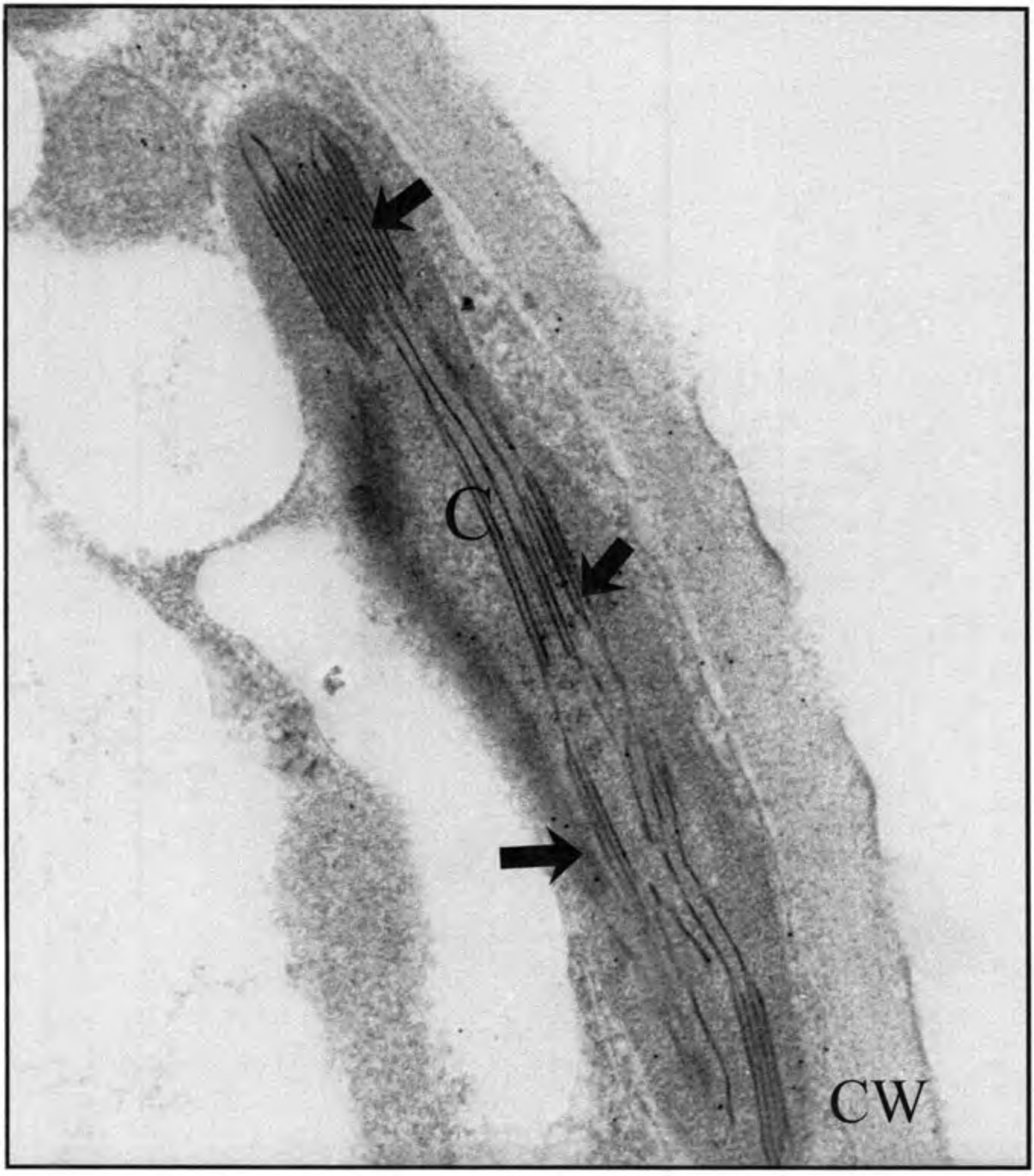

Figure 12: - Transmission electron micrograph showing unspecific labelling in the chloroplast (C) of leaf tissue at 100% RWC embedded in LR White resin. Arrows point to gold particles. antibody conc.=pure, gold conc.=1:50. (x20000)

A Western Blot analysis, using the αTIP antibody, was performed by Clare van der Willigen in order to determine whether the antibody was specific to the proteins that it was supposed to be targeting. The results of this analysis showed a great deal of background attachment to the cellulose membrane used for the western blot as well as specific bands for the proteins that it should target (Fig. 13). This result explains the unspecific binding that I was seeing in the cell wall and chloroplasts. The antibody was binding to the cellulose in these structures as well as the proteins in the tonoplast membranes.

Control grids were viewed. For the first control, the primary antibody was excluded from the labelling procedure and for the second control; the secondary antibody (the gold label) was excluded from the labelling procedure. Both controls were done using leaf tissue at 100% RWC fixed with osmium and embedded in Spurr's resin. No gold particles were seen on the control were the secondary antibody was excluded (not shown) but a few gold particles were found on some parts of the grids where the primary antibody was excluded (Fig. 14). This suggests that some of the unspecific labelling can be attributed to the gold label itself.

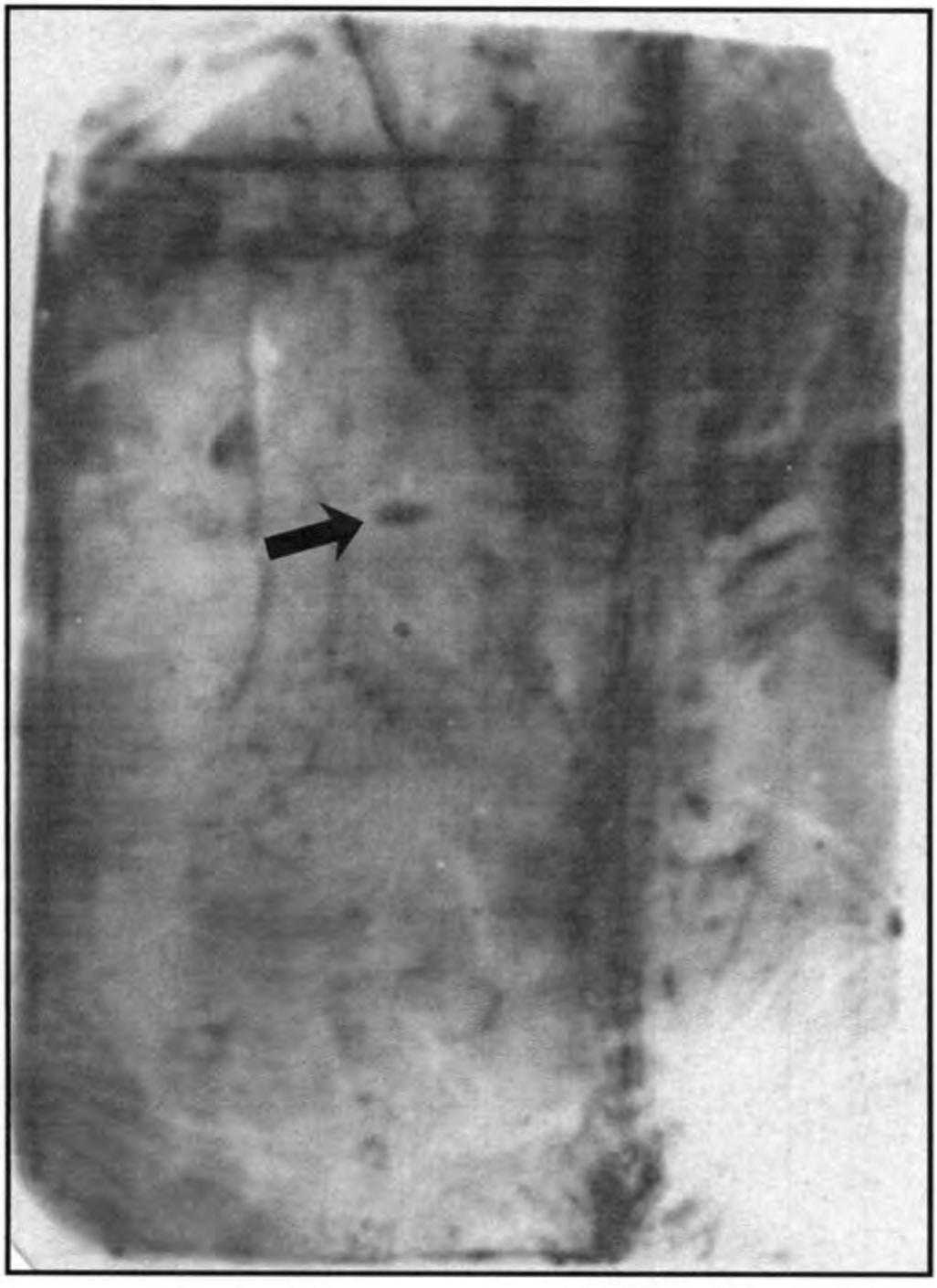

Figure 13: - Western Blot analysis of alpha TIP antibody.
Arrow points to protein band. Dark areas indicate areas of
background attachment to the cellulose membrane by the antibody.

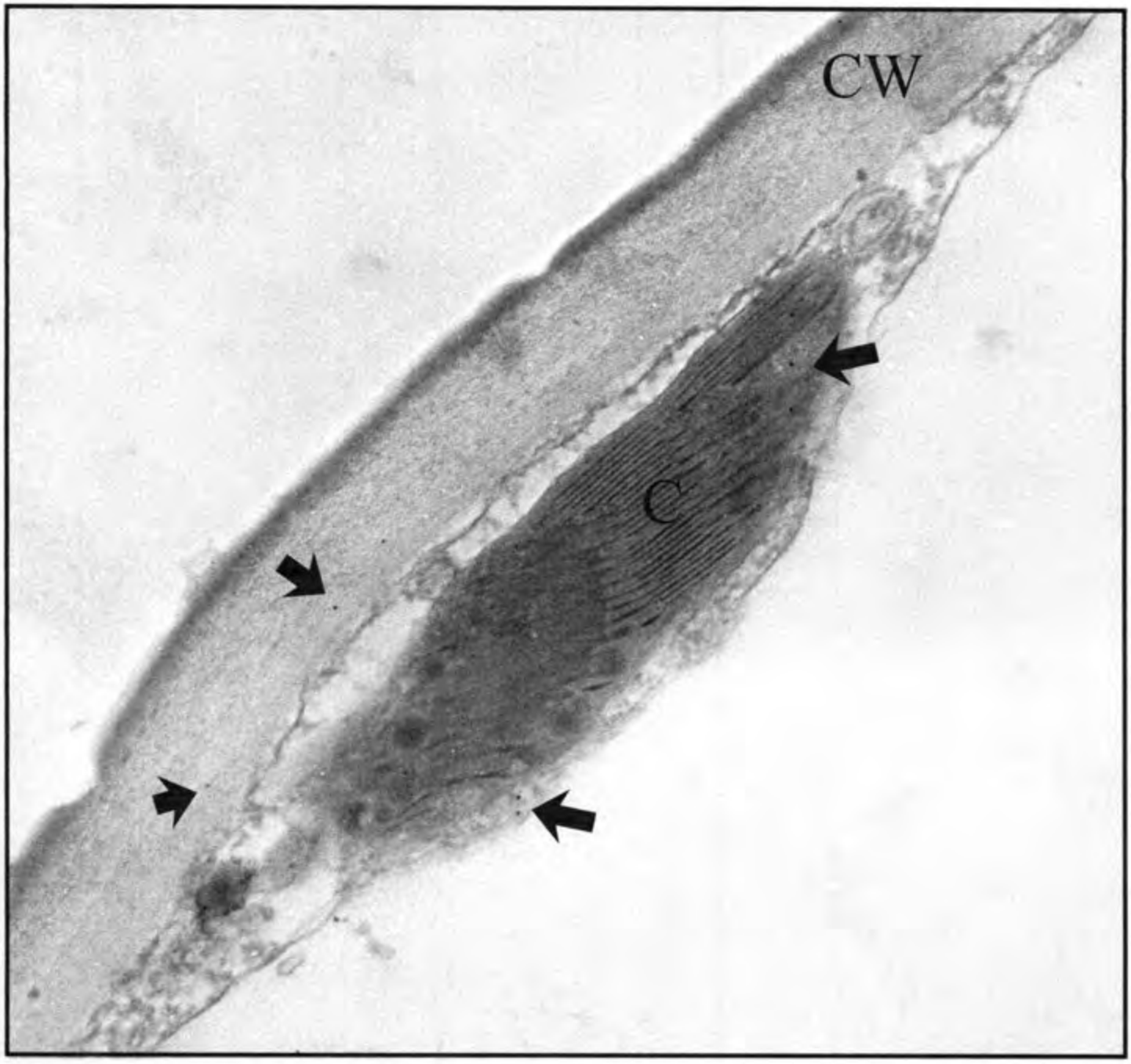

Figure 14: - Transmission electron micrograph showing unspecific gold particles in the cell wall (CW) and chloroplast (C) of leaf tissue at 100% RWC in which no primary antibody was used (control 2). Arrows point to gold particles. gold conc.= 1:50. (x20000)

It was decided to try and count the number of gold particles on the tonoplast in tissues at 100% and 25% RWC while ignoring those that are unspecific (i.e.: - any on the cell wall or in the chloroplast). This would give an indication of whether the number of TIPs does change between different water contents. The samples were labelled with δ TIP antibody at a concentration of 1 in 2 and a gold concentration of 1 in 50. When viewed they showed unspecific labelling as expected (Figs. 15, 16 and 17), however there were insufficient gold particles on the tonoplast (Fig. 17) to make a comparison between the levels found in 25% and 100% samples.

The conclusion arrived at after testing was that the unspecific labelling seen on all the grids viewed was caused by a combination of factors. These factors are that the antibodies labelled walls and chloroplasts unspecifically and were possibly not well suited to electron microscope work. The labels did not bind to the tonoplast membrane, possibly due to insufficiently specific antibodies, fixation of sections using osmium or use of Spurr's resin in embedding procedure. The affinity of the antibody for binding to cellulose and the few unspecific gold particles deposited by the gold label itself also accounted for the unspecific labelling seen in the cell wall and chloroplast. I think that this project is worth pursuing using different techniques. Unfortunately due to time constraints I was not able to test ways of overcoming the problem of unspecific labelling but I will discuss some possible methods that could be used.

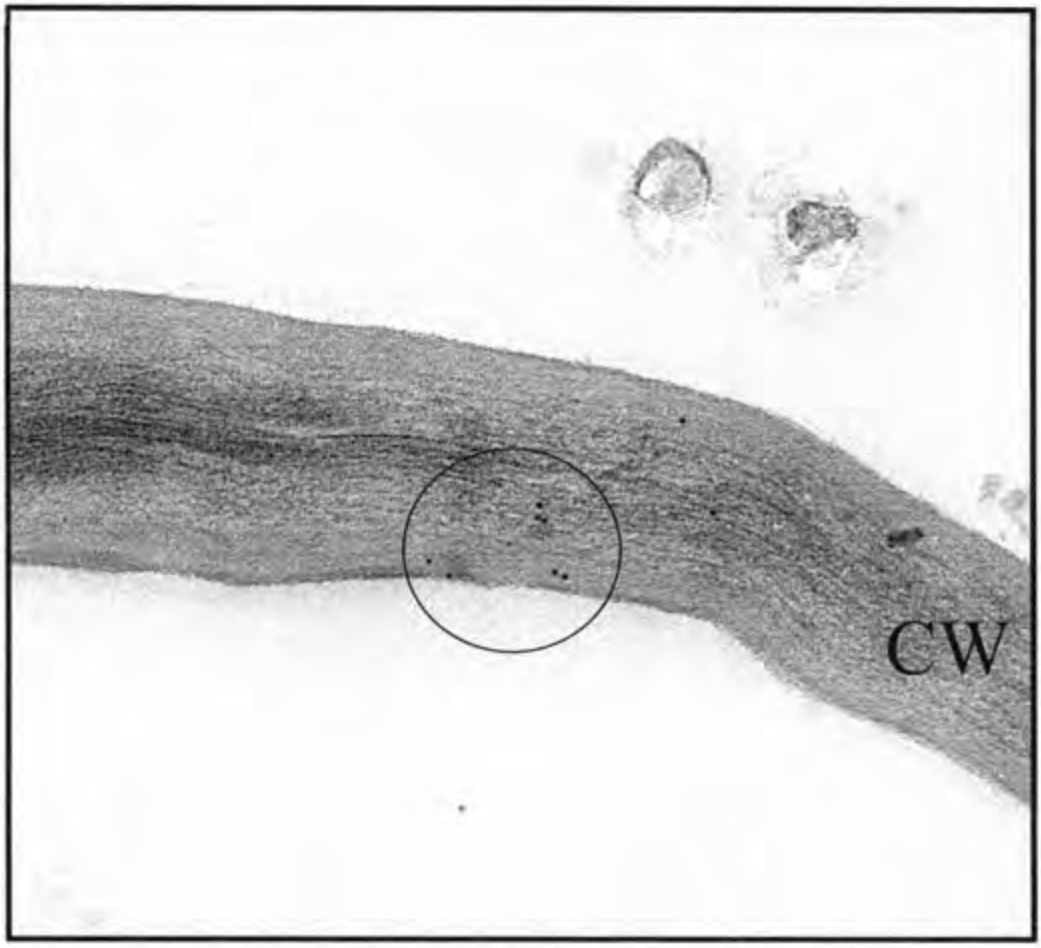

Figure 15:- Transmission electron micrograph showing unspecific labelling in the cell wall (CW) of leaf tissue at 25% RWC. Group of gold particles is circled. antibody conc.=1:2, gold conc. = 1:50. (x30000)

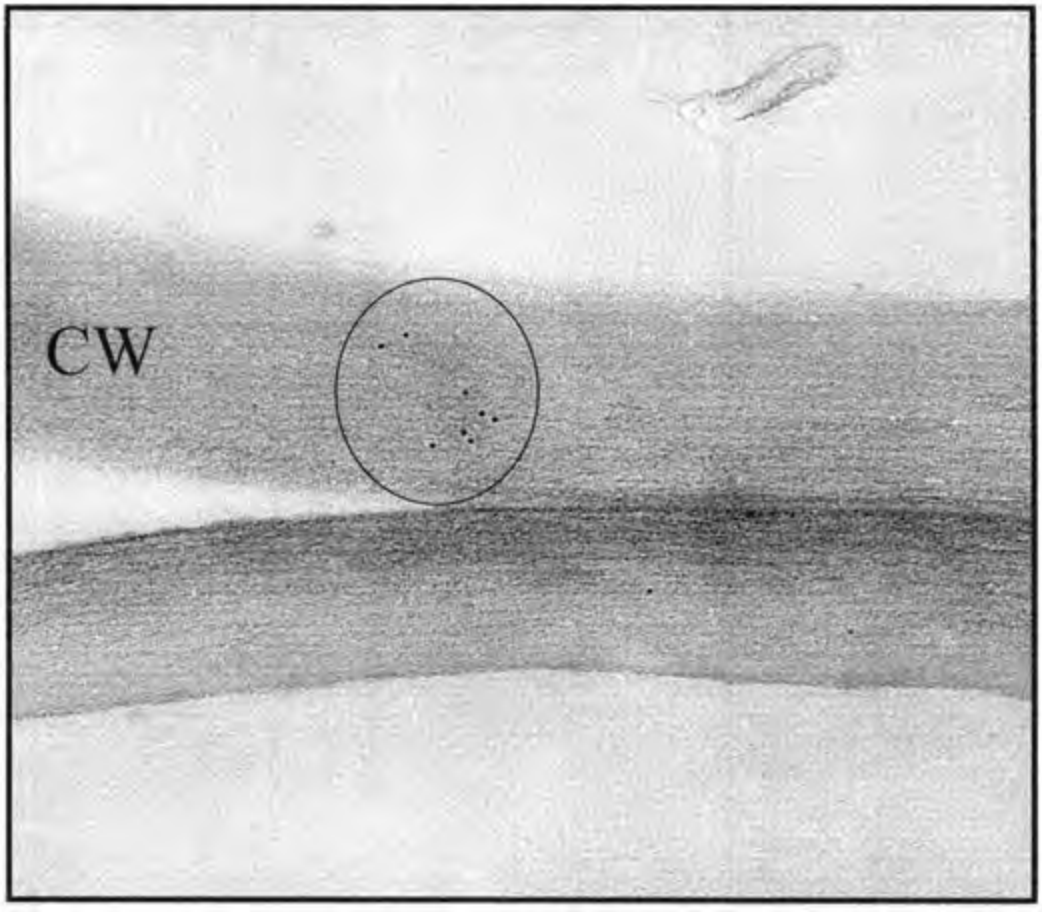

Figure 16: - Transmission electron micrograph showing unspecific labelling in the cell wall (CW) in leaf tissue at 25% RWC. Group of gold particles circled. antibody conc.=1:2, gold conc.=1:50. (x30000)

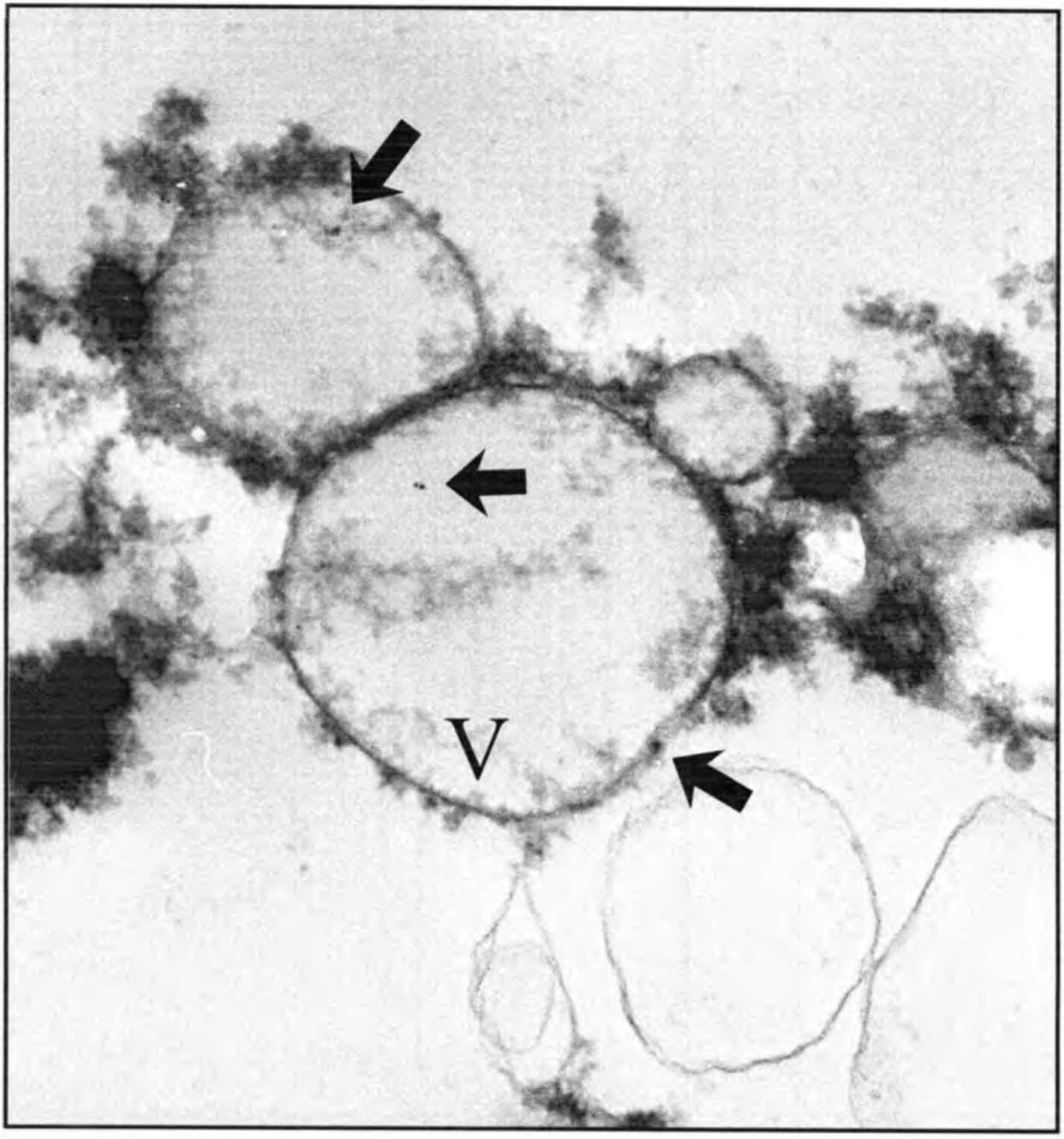

Figure 17: - Transmission electron micrograph showing unspecific gold particles in vacuoles (V) in leaf tissue at 25% RWC. Arrows point to gold particles. antibody conc.=1:2, gold conc.=1:50. (x20000)

A possible method of eliminating unspecific labelling would be to remove the cellulose from the sample after fixing so that the cause of unspecific labelling in the cell wall was removed. An enzyme could be used to digest the cellulose in the sample. The main problem with this method is that cellulose forms the basis of the cellular structure of plants; meaning that the sections may be damaged and not viewable after the cellulose is removed.

Another possibility is to find something that blocks binding sites of the cellulose. The samples would be pre-saturated with the agent that binds to cellulose so that when the protein antibody is added it cannot bind to the cellulose as well. Berg *et al* (1988) used gold labels to localise cellulose in plant cell walls. They discovered that commercially available cellulase binds specifically to the cellulose in cell walls. This may be useful to bind to the cellulose in the cell walls and chloroplasts of *Craterostigma plantagineum*, blocking the binding sites on the cellulose and reducing unspecific labelling in the sections.

If sections can be obtained without unspecific labelling an important issue is how to accurately count the gold particles present in the tonoplast. All particles in a section must be counted accurately in order that conclusions can be drawn from their distribution in the cells. Bisland *et al* (1999) outlined a method of quantifying intracellular gold particles using computer-imaging techniques. Using image software programs they quantified both the accumulation and distribution of gold particles in 2 dimensional cell sections.

Electron microscope images are scanned into a computer and analysed using Adobe® Photoshop® in conjunction with the public domain NIH image program (version 1.61). Using various tools associated with Adobe® Photoshop® (Bisland *et al*, 1999) the images are modified allowing the computer to analyse them, giving the number of particles and their density on the section. Areas of the section containing gold particles can be cropped out and any background "noise" resulting from staining of the grids can be removed. Any gold particles outside the area of interest can be removed from the images allowing for more specific analysis of the target area. Clusters of gold particles on a section can be resolved into single objects by varying the amount of contrast and thresholds applied to the image (Bisland *et al*, 1999). This method would be very useful in determining the number of gold particles that bind to the TIPs in the tonoplast as well as efficiently removing the unspecific gold particles from the images. It is faster and more accurate than using the conventional manual counting techniques.

The presence of substances filling the vacuoles during desiccation tolerance has been highlighted as a possible mechanism of reducing mechanical stress on the plant cells during drying (Farrant, 2000). It is possible that TIPs in *Craterostigma plantagineum* do not play as important a role as is in other resurrection plants. There is significant folding of the cell walls in *Craterostigma plantagineum*, which helps prevent mechanical stress to the cell walls (Farrant *et al*, 1999; Farrant, 2000; Hartung *et al*, 1998). This might mean that the filling of the vacuoles with non-aqueous substances that help in mechanical support is not as important in *Craterostigma plantagineum* as in other resurrection plants, which do not have the wall folding mechanisms. It would be advisable to extend this

project to include other resurrection plants where the cell walls do not fold and there is more pronounced vacuolation during drying.

In conclusion, I believe that the eventual results of this project could give an understanding of whether concentrations of TIPs change during drying in desiccation tolerant plants. If TIPs are increased during drying it could indicate an important role in the movement of molecules in and out of the vacuoles during drying. If TIPs are involved in this movement of substances over the vacuole membrane they clearly play an important part in inferring desiccation tolerance on the plant tissue.

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