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TITLE:

Shootward Movement of CFDA Tracer Loaded in the Bottom Sink Tissues of *Arabidopsis*

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KEYWORDS:

CFDA, root-to-shoot, tracer, movement, plasmodesmata, phloem, root-hypocotyl junction

SUMMARY:

The goal of this protocol is to show how to load the CFDA into different sites of the bottom parts of *Arabidopsis*. We then present the resulting distribution pattern of CF in the shoots.

ABSTRACT:

The symplastic tracer 5(6)-carboxyfluorescein diacetate (CFDA) has been widely applied in living plants to demonstrate the intercellular connection, phloem transport and vascular patterning. This protocol shows bottom-to-top carboxyfluorescein (CF) movement in the *Arabidopsis* by using the root-cutting and the hypocotyl-pinching procedure respectively. These two different procedures result in different efficiencies of CF movement: about 91% appearance of CF in the shoots with the hypocotyl-pinching procedure, whereas only about 70% appearance of CF with the root-cutting procedure. The simple change of loading sites, resulting in significant changes in the mobile efficiency of this symplastic dye, suggests CF movement might be subject to the symplastic regulation, most probably by the root-hypocotyl junction.

INTRODUCTION:

Many fluorescent tracers with a range of spectral properties, such as 5(6)-carboxyfluorescein (CF)[1], 8-hydroxypyrene-1,3,6-trisulphonic acid[2], Lucifer yellow CH (LYCH)[3], Esculin and CTER[4], have been developed and applied in plants to monitor symplastic movement and phloem activity. Generally, a symplastic dye is loaded into a cut in the target tissue and the sequential dispersion of the reporter into other parts of plant will demonstrate the intercellular communication.

Although the mechanism of dye absorption is not fully understood, the principle underlying CF movement inside live cells has been widely acknowledged. The ester form of CF (CF diacetate, CFDA) is non-fluorescent, but membrane-permeable. This property allows rapid membrane diffusion of the dye into cells. Once inside live cells, intracellular esterases remove the acetate groups at the 3' and 6' position of CFDA, releasing the fluorescent and membrane-impermeable CF (**Figure 1**, alternatively refer Wright et al.[2]); CF can then move through the plasmodesmata to other parts of plants.

A well-established procedure with CFDA is that it can be loaded into source leaves and used to monitor the phloem streaming and phloem unloading in the sink tissues of many species, e.g., as CF unloading in the *Arabidopsis* root[5], phloem unloading during potato tuberization[6], phloem unloading in the *Nicotiana* sink leaves[7], and so on. By similar loading approaches, other studies have adopted this dye to demonstrate the symplastic connection between host and parasite[8,9], or to reveal the symbiotic relationships[10,11].

Another way to make use of this dye is to load it into specific cells or single cell by microinjection to determine its distribution pattern. Such sophisticated techniques have greatly facilitated our deeper understanding of plasmodesmata-mediated intercellular communication, particularly in the development of the concept of symplastic domain[12,13]. For example, the microinjection of CFDA into cotyledon cells of *Arabidopsis* resulted in the dye-coupling pattern in the hypocotyl epidermis but non-coupling in the underlying cells or in the root epidermis, therefore the hypocotyl epidermis forms a symplastic domain[14]. Similar domains, such as the stomatal guard cells[15], sieve element-companion cells[16], root hair cells[14] and root cap[17,18] have been identified by microinjection technique. Most surprisingly, some domains allow tracer molecules to move in a certain direction. Take the trichome domain for example, microinjection of a fluorescent probe into the supporting epidermal cell leads to the flow of tracer into the trichome domain, however, the reverse injection does not hold true[19]. A recent report has also found similar situations in the symplastic domains of the *Sedum* embryo[20]. Thus, all above cases imply that swapping of loading sites may lead to novel insights into symplastic communication. Our previous experiment aiming to dissect the route of root-to-shoot mobile silencing identified a novel symplastic domain, or the HEJ (Hypocotyl-epicotyl junction) zone, which was further verified through the root-loading (non-canonical sink-loading) CFDA experiment[21]. Here, we further elaborate the root-to-shoot CF movement by using a simple method and recover a potential symplastic domain by shifting the loading sites. Furthermore, this procedure may be adapted to differentiate genetic backgrounds that have altered root-to-shoot long-distance transport.

PROTOCOL:

1. *Arabidopsis* vertical growth in MS medium

1.1. The interior of laminar flow cabinet needs to be treated with 30 min UV light and 15 min airflow before usage. Make sure to close the glass door when UV light is on. Spray all tools and plates with 70% ethanol before placing them in the cabinet.

1.2. Prepare the Murashige and Skoog (MS) medium in a standard 90 mm-diameter Petri dish under a laminar flow cabinet.

NOTE: The MS medium contains the following components: 20.6 mM NH_4NO_3, 18.8 mM KNO_3, 1.25 mM KH_2PO_4, 1.5 mM $MgSO_4 \cdot 7H_2O$, 3 mM $CaCl_2 \cdot 2H_2O$, 0.1 mM $MnSO_4 \cdot H_2O$, 1.03 µM $Na_2MoO_4 \cdot 2H_2O$, 0.1 mM H_3BO_3, 30 µM $ZnSO_4 \cdot 7H_2O$, 0.1 µM $CuSO_4 \cdot 5H_2O$, 0.1 µM $CoCl_2 \cdot 6H_2O$, 1.39 µM KI, 97 µM FeSO4·$7H_2O$, 114.5 µM ethylenediaminetetraacetic acid (EDTA), 4.07 µM nicotinic Acid, 2.44 µM pyridoxine HCl, 0.15 µM thiamine HCl, 2.68 mM glycine, 555 µM myo-inositol, 87.7 mM sucrose and 10 g/L agar.

1.3. Moisturize the sterilized tips or toothpicks by touching the tip or toothpicks to the MS medium, then dip the sterilized seeds one by one onto the fixed position of each Petri dish indicated by the seeding card.

1.3. Seal the Petri dish with paraffin film and sticky tape and place it vertically on a clear stand in a growth room (22 °C, 70% moisture) with a day cycle of 16 h of light and 8 h of darkness (lighting from 6 am to 10 pm). The *Arabidopsis* plant is ready for CFDA loading on day 9–13 after sowing.

NOTE: The following procedure is performed in the afternoon from 2 pm to 5 pm.

2. CFDA loading with the root cutting procedure

2.1. Prepare fresh CFDA working solution immediately before use. Dilute the 1 mM CFDA stock solution stored in -20 °C freezer with sterile ultrapure water to a working concentration of 5 µM.

2.2. Cut the micro-porous paraffin membrane film (see **Table of Materials**) into small pieces of 3 mm x 3 mm size.

2.3. Uncover the growing plants in the room at 22 °C and clear the excess moisture with a paper towel. Place the small film pieces below each root.

NOTE: From this to step 2.6, the whole process should be completed within 15 min.

2.4. Lift the plants onto the Petri dish lid. Cut the roots in a position about 5–10 mm below the root-hypocotyl junction. Place the shoot part back on to the film on the medium.

2.5. Carefully apply 0.25 µL of CFDA onto the cutting end of each root under a dissecting microscope. Avoid splashing to other parts of plant.

2.6. Cover the plate and leave the plants under light for 2–3 h (22 °C) in the growth room.

2.7. Rinse the stained plants three times sequentially in three separate beakers filled with distilled water, then observe the plants under a stereo-fluorescence microscope with zoom from 1.4x to 3.3x (see the **Table of Materials**). For fluorescence detection, use a high efficiency filter cube

(470/20 nm excitation, 495 nm split and 525/50 nm emission) mounted to transmit the fluorescence signal.

3. CFDA loading with hypocotyl-pinching procedure

3.1. Prepare fresh CFDA working solution immediately before use. Dilute the 1 mM CFDA stock solution stored in -20 °C freezer with sterile ultrapure water (see the **Table of Materials**) to a working concentration of 5 µM.

3.2. Cut the micro-porous paraffin membrane film (see **Table of Materials**) into small pieces of 3 mm x 3 mm size.

3.3. Uncover the growing plants in the room at 22 °C and clear the excess moisture with a paper towel.

NOTE: From this to step 3.7, the whole process should be completed within 15 min.

3.4. Lay a small piece of film under the root-hypocotyl junction of each plant.

3.5. Use forceps to gently pinch the hypocotyl near to the root-hypocotyl junction under a dissecting microscope.

3.6. Carefully apply 0.1 µL of CFDA onto the wound site under a dissecting microscope. Avoid splashing to other parts of plant.

3.7. Cover the plate, and leave the plants under light (22 °C) for 2–3 h.

3.8. Rinse the stained plants three times sequentially in three separate beakers filled with distilled water, then observe the plants under a stereo-fluorescence microscope with zoom from 1.4x to 3.3x (see the **Table of Materials**). For fluorescence detection, mount a high efficiency filter cube (470/20 nm excitation, 495 nm split and 525/50 nm emission) to transmit the fluorescence signal.

REPRESENTATIVE RESULTS:

Symplastic movement is often subject to environmental fluctuations. Perturbation of the plant growing state, and even the process of tissue preparation will affect the size exclusion limit of plasmodesmata, thus affecting the symplastic transport[22]. To improve the staining efficiency, we confine our operation in the growth room, where the temperature and moisture is tightly controlled, and also perform the whole procedure as quickly as possible (ideally within 10–15 min after lifting the lid of Petri dish). These precautions during an experiment can effectively reduce the rates of unsuccessful shoot staining.

We described two slightly different procedures to demonstrate CF shoot-ward movement. Normally, both procedures can lead to CF staining in the shoots about 2 h after feeding (**Figure 2**). Nevertheless, the two procedures produce different staining efficiencies. The hypocotyl-

pinching procedure results in 91% staining efficiency, whereas the root-cutting procedure produces 70% (Welch's *t*-test, $p < 0.001$) (**Figure 3**). We also tried loading the dye by a root-pinching method and found an even lower staining efficiency compared with root-cutting method, suggesting that the approach to load the dye in the root does not account for the staining difference between the root and hypocotyl loading sites (**Figure 3**). The CF signal is mainly found in the vasculature, but only few plants show the half-leaf pattern (**Figure 2**) as seen in other macromolecule movement patterns[21]. Once the CF signal is spread to the shoot, it can be maintained for more than 72 h and the signal cannot unload further to other cell types; this is consistent with previously published results[17].

FIGURE LEGENDS:

Figure 1: A schematic illustration for CFDA uptake and CF movement in the plant's cells.

Figure 2: CF signal in the shoots of 9, 11 and 13 day after sowing (DAS) *Arabidopsis* plants. CF signal can be detected in both cotyledons and true leaves (**A**, **B**, **C**). In the majority of plants, the CF signal is observed in the vasculature after 2–3 hours of loading with either the root-cutting or hypocotyl-pinching method. Only in a very rare cases (less than 0.5%), the hypocotyl-pinching method can generate a partial staining pattern in the cotyledon of 7 DAS Arabidopsis (**D**).

Figure 3: The staining efficiencies with the root-loading method (root-cutting and root-pinching) and hypocotyl-pinching method. The staining efficiency in 9 DAS plants was determined in three independent experiments ($n = 26$ in the root-pinching experiment; $n = 335$ in the root-cutting experiment; $n = 522$ in the hypocotyl-pinching method). Error bar indicates standard error. *** indicates $p < 0.001$.

DISCUSSION:

Emerging studies have shown that plants can rapidly respond to external stimuli[23], including manipulation introduced to the experimental procedures[22]. In our initial experiment, our oversight of this knowledge often leads to staining failure. With these lessons, we suggest that the following precautions should be kept in mind when performing this experiment: (1) the seeds after harvest should be kept in a storage cabinet set to a low temperature and low moisture; (2) manipulation of plants, particularly the exposure to air in the cabinet, should be kept to a minimum time; (3) the experimental conditions should be kept constant, e.g., all the procedures should be performed in a growth room.

Another aspect in this experiment that needs to be pointed out is that the loading volume of CFDA should be kept as small as possible. Excess solution often leads to artifacts in which the excess CFDA solution can diffuse up to the shoot through capillary action, thereby tinting the trichomes of young sink leaves. Although a washing step before imaging can diminish this artifact, the best approach is to load a minimum amount to avoid complication caused by excess solution.

With these technical precautions resolved, the root-to-shoot movement of CFDA can be stably observed as shown in **Figure 2**. When plants grow older, say over 24 days, the *Arabidopsis* plants

seem to lose the ability to transmit CF to the shoot, for which we have not yet found an exact explanation. One possible clue comes from its intracellular accumulation. According to the reports by Wright et al.[2], CF is liable to be sequestrated by vacuoles, therefore, the intracellular free CF over the course of translocation reduces gradually to sequestration in the larger vacuoles of aging plants.

One obvious feature of this procedure is the distribution pattern of CF in the shoot. This vascular pattern is reminiscent of those by loading the dye in the source leaf[1,24,25], thus leading to an illusion that the dye also moves through the phloem. In fact, the bottom-to-top translocation of CF may not be achieved through the phloem given the fact that the root is a strong sink tissue and the shootward movement of CFDA goes against phloem streaming. Rather, this process may be facilitated by xylem transport as shown by Botha et al.[25]. Briefly, CFDA can be taken up through xylem vessels, processed in parenchyma cells and further translocated to the sieve element of phloem stream[25]. Therefore, the loading experiment in this way may not reflect the activity of phloem, but the symplastic movement of CF must occur as the strong fluorescence can be detected. In other words, this bottom-to-top CF movement may result from the combined xylem transport and symplastic transport through parenchyma cells.

Symplastic transport is often subject to symplastic domain formation[13], and in certain circumstances it displays unidirectional transport[19,20]. One way for a quick check is to shift loading sites. Indeed, when we elaborated this process using the same method, we found the simple change of loading site, from root side to the hypocotyl side proximal to root-hypocotyl junction, would result in significant change of CFDA mobile efficiency (**Figure 3**). The CF mobile differentiation due to the distinct loading site would suggest that the root-hypocotyl junction is another symplastic domain, where the symplastic barrier is formed for the root-derived shootward signals. Further experimental design with other molecules is needed to explore this possibility.

So far, this simple method can provide stable shootward movement of CFDA. This feature can be further explored to distinguish plants with compromised or enhanced root-to-shoot movement which has seldom been studied.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 3

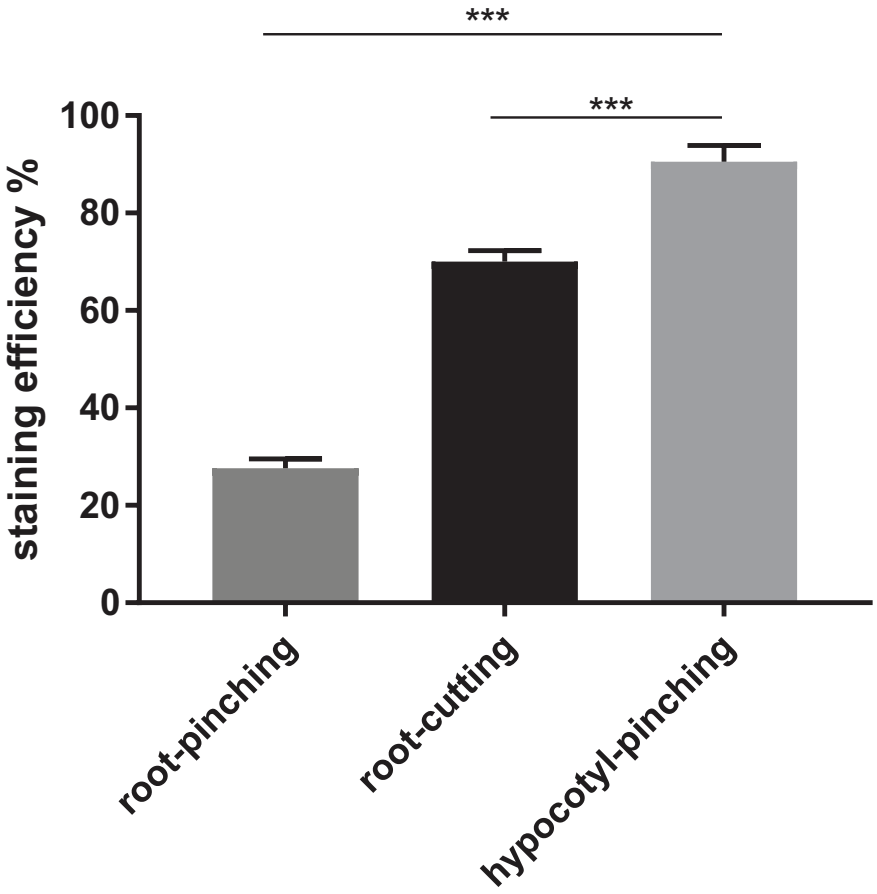

Figure 1

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Figure 2

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Name of Material/Equipment	Company	Catalog Number	Comments/Description
KNO_3	Sinopharm Chemical Re	10017218	
KH_2PO_4	Sinopharm Chemical Re	10017608	
$MgSO_4 \cdot 7H_2O$	Sinopharm Chemical Re	10013018	
$CaCl_2 \cdot 2H_2O$	Sinopharm Chemical Re	20011160	
$MnSO_4 \cdot H_2O$	Sinopharm Chemical Re	10013418	
$Na_2MoO_4 \cdot 2H_2O$	Sinopharm Chemical Re	10019818	
Boric Acid	Sinopharm Chemical Re	10004818	
$ZnSO_4 \cdot 7H_2O$	Sinopharm Chemical Re	10024018	
$CuSO_4 \cdot 5H_2O$	Sinopharm Chemical Re	10008218	
$CoCl_2 \cdot 6H_2O$	Sinopharm Chemical Re	10007216	
KI	Sinopharm Chemical Re	10017160	
$FeSO_4 \cdot 7H_2O$	Sinopharm Chemical Re	10012118	
EDTA	Sinopharm Chemical Re	10009717	
NaOH	Sinopharm Chemical Re	10019718	
KOH	Sinopharm Chemical Re	10017018	
Sucrose	Sinopharm Chemical Re	10021418	
Myo-inositol	MACKLIN	I811835	
Nicotinic Acid	MACKLIN	N814565	
Pyridoxine HCl	MACKLIN	V820447	
Thiamine HCl	MACKLIN	T818865	
Glycine	MACKLIN	G800880	
Agar powder	Novon	ZZ14022	
Fluorescence Microscope	Zeiss	Axio Zoom V16	
Dissecting microscope	SDPTOP	SRE-1030	
200µl pipette	Dragon Laboratory Instr	713111110000-20-200ul	
2.5µl pipette	Eppendorf	3120000011	
Fine forceps	TWEEZERS	ST-15	
Parafilm	PARAFILM	PM-996	
Stainless steel double-sided blade	Gillette	Platinum-Plus Double-Edge Blade	

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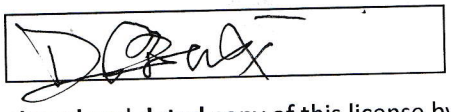
Signature:	
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Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

Thanks editor for giving me this opportunity to improve this manuscript. We have gone through the whole manuscript and found no spelling or grammatical errors.

• Please use title Case of the manuscript title in the video and manuscript: Shootward Movement of CFDA Tracer Loaded in the Root and Hypocotyl of Arabidopsis

Yes. A correction has been made in both video and manuscript.

• Abstracts: Please remove citations from the abstract.

Yes, they are deleted.

• Introduction: Please expand your Introduction to include the following: The advantages over alternative techniques with applicable references to previous studies; Description of the context of the technique in the wider body of literature; Information that can help readers to determine if the method is appropriate for their application.

Yes, I have expanded the introduction as you suggested. Please see the highlighted in the text.

• Protocol Detail:

1) 2.6: Under light?

Yes.

2) 2.7: Mention magnification

Yes, included

3) Please ensure that all details present in the video are also mentioned in the manuscript (e.g., moisture removal at 3:03 is not present in the manuscript)

Yes. These details have been now included.

4) Please ensure homogeneity between the video and the manuscript.

The text has been rearranged to match the video.

• Discussion: JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please expand the discussion to cover the following in detail (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

Yes, all the contents related to above 5 problems have been added in the discussion. Please see the text.

• Figures:

1) Please remove the embedded figures from the manuscript. Figure legends, however, should remain within the manuscript text, directly below the Representative Results text.
   Yes. Done.
2) Fig 2: Add scale bars
   Yes. Added.
3) Fig 3: define the error bars, and ***.
   *Yes, included "Error bar indicates standard error. *** indicates p<0.001."*
4) Please remove the figure/table legends from the figure files and place them directly below the Representative Results text.
   Done

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Yes. I now changed the reference style to comply with JoVE.

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Yes. The parafilm has been changed to "micro-porous membrane film". Milli-Q water has been replaced with ultrapure water. ZEISS Axio Zoom V16 has been replaced with stereo fluorescent microscope.

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Figure 1 was generated in this manuscript. We explicitly converted the rationale (according to the description by Wright et al. (1996) [1] into the Figure to facilitate understanding of this protocol.

• Video Comments:

• 0:05-0:08 - The narration audio is weighted heavily to the left stereo channel during this time, before shifting to a balanced mix. This should be corrected.

Corrected.

• All audio should be peaking between -6 and -12 dB. The audio in this video seems low. The audio volume should be raised.

Yes, the volume was raised to ~7 dB.

• 1:21 - It seems like there is a hard edit in the middle of this fade. This should be corrected.

Corrected.

• 1:31, 2:50, 3:22, 4:25 - The edits here are jump cuts, which tend to have a jarring effect on the viewer. They should be smoothed out with crossfades instead.

Corrected.

• 2:48 - The shot of the parafilm packets either needs to be extended or removed. It is currently not on screen long enough, and quickness of the edits is disorienting.

Yes, corrected.

• 5:39 - A title card should be inserted here that reads, "Conclusion".

Done.

Comments from Peer-Reviewers:

Please note that the reviewers raised some significant concerns regarding your method and your manuscript. Please revise the manuscript to thoroughly address these concerns. Additionally, please describe the changes that have been made or provide explanations if the comment is not addressed in a rebuttal letter. We may send the revised manuscript and the rebuttal letter back to peer review.

Yes. We have addressed all the issues raised by the reviewers. Please check the following explanations.

Reviewer #1:

Manuscript Summary:

Review: "Shootward movement of CFDA tracer loaded in the root and hypocotyl of Arabidopsis"

This manuscript addresses the delivery of a phloem-specific fluorescent tracer, carboxyfluorescein, for acropetal translocation. To enhance delivery of the membrane-permeant formulation carboxyfluorescein diacetate (CFDA), the authors examine the movement of the fluorescent tracer into the shoot following application to cut roots or pinched hypocotyls.

The video submitted with this manuscript is very clear, easy to understand, and demonstrates the root cutting and hypocotyl pinching methods meticulously.

Major Concerns:

The manuscript is very brief, lacks depth, and contains problems which lead me to question the value of these approaches.

1) I would expect transported CF to be detected in sink tissues. But the newly emerged true leaves have no signal at 9 DAS and indeed CF staining is weakest in the youngest sink leaves at later developmental stages. Why is this the case? Is this consistent with phloem unloading, or do these observations more likely reflect xylem transport? My concerns continue with the suggested "half-zone pattern": the "partially staining pattern of the cotyledon" is nonsensical as it is not vascular and thus is not translocated CF. It is misleading to describe this result as similar to gene silencing as there is no gene being silenced, rather a chemical is delivered, and it dismisses the problem.

   Thanks for pointing out this issue. Indeed, the signal in the newly formed leaf is quite weak. We have provided the explanation in the discussion (please see the text). Basically, our explanation is that it is a mixed translocation of both xylem and parenchyma cells-*facilitated symplastic transport, which was shown by Botha et al. (2008)[2]. We suggest that the CFDA was first transported through xylem. Then, it is unloaded from xylem into mature leaf that needs water most to support photoassimilate synthesis. Because we can*

observe the fluorescence signal, meaning it must move through the live cells, most probably through parenchyma cells*. As for the half-leaf mobile pattern, it is possibly a very tightly-regulated movement. We rephrased the sentence to avoid misunderstanding.*

2) As emphasized in Fig. 1 and found in the Introduction (line 48), the membrane permeant carboxyfluorescein diacetate (CFDA) is cleaved by endogenous esterases, yielding membrane impermeant carboxyfluorescein (CF). This is not new information, indeed it is fundamental to the use of this fluorescent tracer. Thus, references are appropriate, and I think its inclusion in Fig.1 is misleading as it is not a result from this work.

Fig. 1 was regenerated from the description by Wright et al. (1996)[1]. Indeed, it's not new, nevertheless it may, as you pointed out, help reader to easily take the whole message.

3) The methods report pipetting 0.1 µL (line 74) in the hypocotyl pinching procedure. How does one pipet this volume accurately? I think suggesting a larger volume to pipet of a diluted stock would yield more reproducible results.

Thanks for pointing this out. Actually, we should keep the volume as low as possible. The Eppendorf pipette is used at its limit to pick this volume. It might not so accurate, but it works as too much CFDA solution can diffuse up to the shoot through capillary action, thus tinting the young sink leaves. However, keeping the low volume will greatly reduce such capillary effect.

4) n10X different volumes of 5 µM CFDA are delivered to applied to cut roots and pinched hypocotyls. I question how these two approaches can be compared side-by-side when the amount of the tracer applied is so different.

Thanks for raising this critical concerns. As I explained in the last question, we need to keep the loading volume as small as possible. Since roots are far away from the shoot, the quantity of CF is higher needed for its upward movement. Thus, the higher volume loading in the roots is to avoid the observed difference that might come from the lack of sufficient CFDA loaded in the roots. Indeed, if we lower the volume loading, this difference can be more obvious seen in Figure 3. Therefore, we need to provide sufficient CFDA to reveal the real effects that comes from the symplastic domain. Nevertheless, we reduced the amount to 0.25 ul still generate similar effects see in the Figure 3.

Reviewer #2:

The manuscript "Shootward movement of CFDA tracer loaded in the root and hypocotyl of Arabidopsis" (JoVE59606) submitted by Mengting Jiang, Zhuying Deng, Rosemary G. White, Tianlin Jin and Dacheng Liang describes a protocol to demonstrate the bottom-to-top movement of a fluorescent tracer in Arabidopsis by using two approaches, root-cutting and

hypocotyl-pinching. This protocol is useful to plant scientists studying symplastic transport. However, the text is missing some critical details and needs significant editing.

Title

The title describes the assay, but I think that for this protocol a more general title would be appropriate (i.e. there is no need to include the different loading sites for the tracer in the title).

Thanks for your suggestion. The title has been changed as " Shootward Movement of CFDA Tracer Loaded in the Bottom Sink Tissues of Arabidopsis"

Introduction:

The authors should consider expanding the introduction to include more background about types of intercellular transport and approaches used to study those, including advantages and limitations.

Thanks for raising this issues. I have expanded the introduction as you suggested. Please see it in the introduction. The limitations have been addressed in the discussion.

Line 43. The statement "the CF movement inside live cells has been adequately appreciated" is unclear, please rephrase.

Yes. It has been rephrased as "the principle underlying CF movement inside live cells has been widely acknowledged."

Line 50. To which previous experiment are the authors referring to? Please, provide a citation.

Yes. It was now cited.

Line 51. What is a "gating barrier4"? Please, revise the text so that necessary definitions are included and it is easy for scientists who have not conducted this experiment before to follow.

The whole sentence has been rephrased. To make it clear, we added more details in.

Protocol:

The protocol is missing cautionary notes where appropriate (i.e., what steps should take place in a laminar flow hood).

Yes. A note has been added. (Note: The interior of laminar flow cabinet needs to be treated with 30 min UV light and 15 min airflow before usage. Make sure to close the glass door when UV light is on. Spray all tools and plates with 70% ethanol before placing them in the cabinet.)

It is also missing details. For example, a dissecting microscope is listed in the materials table but it use is not mentioned in the protocol.

It's now referred to the protocol. Please see step 2.5, 3.5 and 3.6.

Step 1.1. From the materials list it is clear that a non-commercial MS mixture is used, and the recipe to prepare the MS medium should be included in the protocol. Also, include the agar concentration utilized.

MS recipe and agar concentration has been uncluded in step 1.1

Step 2.6: Should the plates be placed vertically also after this step?

Thanks for making this suggestion. We have indeed done this and it does not make a difference. Thus, to keep it simple and avoid plants tipover, we keep plates horizontal.

Step 2.7. How are the samples prepared for microscopy?

Once the excess dye was washed off, the plants can be directly observed in the stereo microscope. No additional preparation was needed.

Step 2.7. Provide all the microscope settings used to observe the plants and acquire the images (i.e., light source, filters, etc.). Same comment applies to step 3.6.

This information has been added.

Results

Line 98. Change "constantly kept" to "tightly controlled".

Yes. changed

Line 99. Cautions should be included within the protocol, not as part of the results.

Yes. these cautions were added into protocol.

Discussion

Lines 136/137. The statement "the bottom to top translocation of CF can be practically achieved if not via phloem" is unclear. Please, rephrase.

To make it clear, we have rephrased the whole paragraph. Please see the discussion.

Reviewer #3:

In the submitted manuscript/protocol addresses the loading of CFDA into A. thaliana seedlings at hypocotyls and cut roots. The authors present and describe nicely the method. In general the protocol is very useful but lacks some key information.

Major concern:

It is not mentioned whether seedlings were grown under dark/nigh cycles and at which time point of the day (end of day, midday?) the loading and imaging was performed. It is also not mentioned what MS medium (with sugar/w/o sugar, concentration of MS, agar ..etc.) was used in the experiments. Water uptake, xylem and phloem streaming, and, thus, of CFDA might I be very different at various day times and also depends on sugar concentrations used in the growth medium. This has to be mentioned and/or might be considered to be addressed experimentally. Another aspect that should be mentioned is that CF accumulates in vacuoles of cells sometime after loading.

> Thanks for your concerns raised here. We did not know whether day/light cycle will affect the dye movement. But any way, we have achieved consistent results. We here provided this piece of information in step 1.3 and 1.4. Step 1.3, (**lighting from 6am to 10 pm.).** step 1.4, "**Note: the following procedure is performed on the afternoon from 2pm to 6 pm."**
>
> Sugar content is also provided in step 1.1. Yes, we included the CF accumulation in the discussion. Please see the text "When plants grow older, say over 24 days, the Arabidopsis plants seem to lose the ability to transmit CF to the shoot, for which we have yet found an exact explanation. But, one possible clue is coming from its intracellular accumulation. According to the reports by Wright et al. (1996), CF is liable to be sequestrated by vacuoles, therefore, the intracellular free CF over the course of translocation is becoming less and less due to sequestration in the larger vacuoles of aging plants."

Minor concerns:

Line 139 correct " CF was showed to move from root to shoot …" to "CF was shown to move …"

Thanks for pointing this out. It was corrected.

Line 51 the reference number "4" should be superscripted.

Yes, corrected.

1 Wright, K. M. & Oparka, K. J. The fluorescent probe HPTS as a phloem-mobile, symplastic tracer: an evaluation using confocal laser scanning microscopy. *Journal of Experimental Botany.* **47** (296), 439-445, (1996).

2 Botha, C. E. J. *et al.* A xylem sap retrieval pathway in rice leaf blades: evidence of a role for endocytosis? *Journal of Experimental Botany.* **59** (11), 2945-2954, (2008).