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### The genetics of vacuoles

*Biogenesis and function in plant cells*

Li, S.

#### Publication date

2020

#### Document Version

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#### Citation for published version (APA):

Li, S. (2020). *The genetics of vacuoles: Biogenesis and function in plant cells*. [Thesis, fully internal, Universiteit van Amsterdam].

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# Summary

Vacuoles are present in the cells of plants, fungi, protists and animals. However, their importance is most pronounced in plants due to the multiplicity of functions and the dimensions of vacuoles in plant cells. An interesting, and relatively recently discovered aspect of plant vacuoles is that some cell types have multiple vacuoles with very different content and functions. This raises questions how multiple vacuoles can be formed in a single cell and how proteins are directed to the one or the other vacuolar compartment.

The identification of vacuolinos, a novel type of additional vacuole that exists besides the anthocyanin containing central vacuole in petal epidermal cells, provided an excellent model system to study the formation and functional differentiation of vacuoles, but not without raising new issues and questions. Each chapter of this thesis is an immersion in the biology of vacuoles, with special emphasis on the biogenesis, maturation and physiology of vacuolinos. Vacuoles are here investigated by a combination of molecular genetics, cell biology and bioinformatics. The results that were obtained provide new insights into the mechanism(s) of regulation of vacuolar pH in extremely acidified cells, the biogenesis and biological function of vacuolinos and the mechanism(s) by which proteins transport from vacuolinos to the central vacuole is controlled

In **Chapter 1**, protein sorting pathways and the biogenesis vacuole in plants and fungi and lysosome in animals, as well as the formation of functionally distinct vacuoles in a single plant cells are reviewed. Comparative analysis of the genes that were shown to be involved in the biogenesis of these compartments suggests both conservation and divergence of the components of the machinery that drives

biogenesis of and protein trafficking to these compartments during evolution in plants, fungal and animal lineages.

The analysis of petunia mutants impaired in vacuolar acidification in the petal epidermis, revealed that vacuolino formation is controlled by the same regulatory network that governs hyper-acidification of the central vacuole where anthocyanins are stored. The transcription factors PH4 (encoding a MYB protein), AN1 (a bHLH), PH3 (a WRKY) and AN11 (a WD repeat protein) together form the MBWW transcription complex. This complex activates the expression of *PH1* and *PH5*, encoding two vacuolar P-ATPases, which hyperacidify vacuoles in petal epidermal cells, conferring a reddish color to the petals, in contrast to the bluish color of mutants affecting this pathway. This very same complex regulates a set of downstream target genes involved in vacuolino biogenesis.

In **Chapter 2**, the machinery responsible for the hyper-acidification of vacuoles in the flesh of *Citrus* fruits is characterized, and shown to be the very same one described for petunia petals. *CitPH1* and *CitPH5*, are expressed in sour lemons, oranges, pummelos and rangpur lime fruits, while their expression is strongly down-regulated in sweet-tasting varieties, which can be traced back to mutations affecting the MBWW complex. Because of the proven role of the MBWW complex in both vacuolar acidification and vacuolino biogenesis, the identification of all components of MBWW complex and the demonstration of its involvement in the expression of *PH1* and *PH5* in *Citrus*, strongly suggests the existence of vacuolino in fruits cells. These considerations open the way to investigate the distribution of vacuolinos in different tissues and different species.

Since their identification, vacuolinos, were shown to be intermediate stations for a range of different proteins on their way to the central vacuole, but the biological

function(s) of these organelles remained largely mysterious, as mutant flowers that lack vacuolinos (e.g. *an1*, *ph4*) do not display any obvious macroscopically visible defects. In specific genetic backgrounds, containing a dominant allele of the *FADING (FA)* locus, loss-of-function *ph4* mutations cause besides the lack of vacuolinos in petal epidermal cell, also the degradation of anthocyanin pigments in the central vacuole and “fading” of the petal color after bud opening. Although the genetics of this phenomenon was described already in the 1980s, the underlying mechanism(s) remained obscure. **Chapter 3** describes the isolation and characterization of the *FADING (FA)* gene, the key factor in the fading process. FA encodes a member of the ferric reductase oxidase (FRO) group of the family of metalloreductase enzymes, and is expressed in petals as well as various vegetative tissues, like leaves and stems.

In petal epidermal cells of mutants for the MBWW complex (*an1*, *ph3* and *ph4* mutants), FA localizes on the tonoplast of the central vacuole where the anthocyanin molecules are stored. These cells lack vacuolinos and FA reaches the central vacuole where it (probably indirectly) destabilizes anthocyanins. In petal epidermal cells of wild type *Petunia* lines, which contain vacuolinos, GFP and RFP-tagged FA protein moves to vacuolinos, just like other vacuolar proteins. Within vacuolinos, FA-GFP (or at least the GFP tag) is released from the membrane and now accumulates in the vacuolinos lumen, which may explain why FA-GFP does not move on from vacuolinos to the central vacuole, as other vacuolar proteins do, and thus cannot trigger the degradation of anthocyanins in there. In the petals epidermis of *ph4* and *ph3* mutants, by contrast, vacuolinos are abolished and FA(-GFP) now reaches the vacuoles, like it does in leaf cells, to trigger the degradation of anthocyanins in there. These findings suggest that vacuolino may serve as a gatekeeper /check point that allows certain proteins to reach the central vacuole, while keeping others away from the central vacuole. Moreover, the new insights in the control of flower (and fruit) pigmentation via the regulation of anthocyanin

degradation, which may be helpful to generate crop varieties with new flower colors or enhanced dietary value of fruits

Previous work showed that the MBWW transcription factor complex is essential for the formation of vacuolinos, MBWW was thought to act indirectly, most like through the activation of downstream genes encoding proteins that are directly involved in membrane trafficking and vacuolino formation. However the pathway by which vacuolinos are formed and the (MBWW regulated) genes and proteins that are involved remained unknown **Chapter 4** presents the identification of the first target gene, *RAB5a*, of the MBWW complex that is required for the biogenesis of vacuolinos. RAB5 is a member of the RAB family of small GTPases, many of which have crucial roles in specifying the “identity” of membranes and the docking and recognition vesicle and target membranes. The identification of RAB5a, a homolog of metazoan RAB5, showed that plant RAB5s are evolutionary more diverse than previously thought and consist of three distinct phylogenetic clades, represented in petunia by RAB5a, RAB5a1, and RAB5a2 plus the homologous (and well-studied) *Arabidopsis* proteins RHA1 and ARA7. The presented data show that the *RAB5a* gene is activated by the MBWW complex and that the RAB5a protein is needed to facilitate the fusion of a subpopulation of prevacuolar compartments (PVCs) to form vacuolinos. Interestingly, RAB5a1 and RAB5a2, localize to different cellular compartments than RAB5a, and cannot functionally substitute RAB5a. This highlights the pivotal role of the RAB5a GTPase in vacuolino formation and provides insights into the diversification of distinct vacuolar compartments during evolution, through duplication and neofunctionalization of genes encoding key regulators of vesicle trafficking.

The abolishment of vacuolinos in *rab5a* mutants, and the formation of enlarged vacuolino induced by expression of *RAB5a* from the constitutive 35S promoter,

affect in *Petunia* the dimension and shape of petal epidermal cells. Such effect on epidermal cells might alter petal coloration and even pollinator preference, and a deeper analysis of the consequences of RAB5a activity on cell structure, might enrich our understanding of the diverse biological function of vacuolino.

Mutants resulting in petal color shift towards blue, defined seven so-called *PH* loci in *petunia*. The loci control hyper-acidification of the central vacuoles in the petal epidermis and thereby the color displayed of the anthocyanins pigments in there, by altering protonation state at different pH. Of these seven *PH* loci, six had been molecularly analyzed previously and their biological functions described. However, *PH7*, remained so far uncharacterized. In **Chapter 5**, comparative analysis of RNA-seq data generated from *PH7* wild type, *ph7* transposon unstable mutant and *ph7* stable mutant have successfully identified the *PH7* gene. *PH7* encodes a monosaccharide sugar transporter that is highly homologous to the *Arabidopsis* ERD6-like6 glucose/proton symporter residing on tonoplast. Mutations in different regions of *PH7* result in distinct defects in the vacuolino pathway, blocking either the formation of vacuolinos (and possibly downstream steps), or the trafficking of proteins from vacuolinos to the central vacuole. This indicates that *PH7* is required at multiple steps along the vacuolino pathway, and that it facilitates the formation of vacuolinos and the subsequent vacuolino-central vacuole trafficking through distinct protein domains and mechanisms. Although, further research is needed to unveil in detail the mechanism underlying the petal color change in *ph7* mutants and the role of this transporter in the physiology of vacuolino membranes, the current findings present evidence that supports previous hypotheses/suggestions that membrane transporters may have additional functions in mediating recognition or fusion of vesicles and target membranes during vesicle and proteins trafficking.

# Samenvatting

Vacuolen zijn organellen die voorkomen in cellen van planten, schimmels, protisten en dieren. De vacuolen in plantencellen zijn het meest bekend, vanwege de veelheid aan functies en de enorme afmetingen van vacuolen in plantencellen. Een interessant en relatief recent ontdekt aspect van plantenvacuolen is dat sommige celtypen meerdere vacuolen hebben met zeer verschillende inhoud en functies. Dit roept vragen op hoe meerdere vacuolen kunnen worden gevormd in een enkele cel en hoe eiwitten worden gericht naar het ene of het andere vacuolaire compartiment.

Vacuolinos, zijn kleine additionel vacuolen recent beschreven in epidermale cellen van bloembladen waar ze voorkomen naast de grote centrale vacuole die de anthocyaan bloemkleurpigmenten bevat. Deze additionele vacuolen vormen een uitstekend modelsysteem om de biogenese en functionele differentiatie van vacuolen te bestuderen. Tegelijkertijd werpen deze vacuolinos ook nieuw vragen op, onder andere met betrekking tot hun biologische functie. Elk hoofdstuk van dit proefschrift is een reis door de biologie van vacuolen, met speciale nadruk op hun biogenese, rijping en fysiologie. Vacuolen worden hier onderzocht door een combinatie van methodes uit de moleculaire genetica, celbiologie en bio-informatica. De verkregen resultaten verschaffen nieuwe inzichten in de mechanismen die ten grondslag liggen aan de regulatie van vacuolaire pH regulatie in extreem verzuurde cellen, de biogenese en biologische functie van vacuolinos en het mechanisme waardoor eiwitten van vacuolinos naar de centrale vacuole worden gestuurd

In **hoofdstuk 1** worden eiwitsorteerroutes en biogenese van vacuolen in planten en schimmels, en lysosomen bij dieren besproken, alsmede de vorming van meerdere functioneel verschillende vacuolen in een enkele plantencel. Analyse en vergelijking van de genen waarvan is aangetoond dat ze betrokken zijn bij de biogenese van deze

compartimenten suggereerde zowel conservering als divergentie tijdens de evolutie van planten, schimmels en dieren van de componenten van de factoren die de biogenese van vacuolen en lysosomen en de sortering van eiwitten naar deze compartimenten reguleert.

De analyse van petunia mutanten met defecten in verzuring van de vacuolen in de epidermis van kroonbladeren (petalen), liet zien dat de biogenese van vacuolino's wordt gecontroleerd door hetzelfde regulatoire netwerk dat de sterke verzuring regelt van de centrale vacuole. De transcriptiefactoren PH4 (coderend voor een MYB-eiwit), AN1 (een bHLH eiwit), PH3 (een WRKY eiwit) en AN11 (een WD-repetal eiwit) vormen samen het MBWW-transcriptiecomplex. Dit complex activeert de expressie van *PH1* en *PH5*, coderend voor twee vacuolaire P-ATPases die vacuolen in epidermale cellen van bloemblaadjes verzuren, en een roodachtige kleur veroorzaken in de bloemblaadjes, in tegenstelling tot de blauwachtige kleur van mutanten die deze route beïnvloeden. Ditzelfde complex reguleert ook een aantal genen die betrokken zijn bij vacuolino-biogenese.

In **hoofdstuk 2** wordt de machinerie gekarakteriseerd die verantwoordelijk is voor de extreme verzuring van vacuolen in *Citrus* vruchten en wordt aangetoond dat deze machinerie dezelfde te zijn als die beschreven voor petunia bloemblaadjes. *CitPH1* en *CitPH5* komen sterk tot expressie in sterk zure citroenen, sinaasappels, pummelo's en rangpur limoenen, terwijl hun expressie sterk is verlaagd in niet-zure vruchten met een zoete smaak. Dit bleek het gevolg van mutaties die de expressie of de activiteit het MBWW-complex beïnvloeden.

In petunia bloemen is het MBWW-complex essentieel voor de expressie van genen betrokken bij vacuolaire verzuring en voor genen die een rol spelen in de vorming van vacuolino's. Analyse van publieke transcriptoom data wees uit dat in zoete sinaasappels, niet alleen expressie van *CitPH1* en *CitPH5* is verlaagd, maar ook de



expressie van *Citrus* homologen van genen die in petunia essentieel zijn voor de vorming van vacuolinos, zoals *RAB5a*. Dit suggereert vacuolinos ook voorkomen in ander weefsels dan de bloembladerepidermis. Deze overwegingen openen de weg om de aanwezigheid en functie(s) van vacuolino in verschillende weefsels en verschillende soorten te onderzoeken.

Vacuolinos zijn kleine vacuole-achtige compartimenten die voorkomen naast de grote centrale vacuole in epidermale cellen van bloemblaadjes. Sinds hun identificatie, bleken vacuolinos tussenstations te zijn voor een reeks van verschillende eiwitten op weg naar de centrale vacuole, maar de biologische functie(s) van deze organellen bleef grotendeels mysterieus. Eén reden is dat bloemen die vacuolinos missen als gevolg van een mutatie (bijv. *an1*, *ph4*) geen duidelijke macroscopisch zichtbare defecten vertonen. Echter in specifieke genetische achtergronden – met o.a. een dominant allel van de *FADING (FA)* locus – veroorzaken *ph4* mutaties naast de afwezigheid van vacuolinos in de bloembladerepidermis, ook de afbraak van de anthocyaan pigmenten in de centrale vacuole. Dit leidt tot het vervagen (“fading”) van de bloemkleur na opening van de bloemknop. De genetica van dit fenomeen werd al in de jaren 1980 beschreven, maar de onderliggende mechanismen waren nog onbekend. **Hoofdstuk 3** beschrijft de isolatie en karakterisering van het *FADING (FA)* gen, de sleutelfactor in het proces van pigmentafbraak. FA codeert voor een lid van de ferro-reductase-oxidases (FROs) uit de familie van metalloreductase-enzymen en komt tot expressie in bloemblaadjes en verschillende vegetatieve weefsels, zoals bladeren en stengels.

In epidermale bloembladcellen van mutanten met een defect MBWW-complex (*an1*, *ph3* en *ph4* mutanten) accumuleert FA op de tonoplast van de centrale vacuole waar de anthocyaan moleculen zijn opgeslagen. Deze cellen missen vacuolinos en FA accumuleert in de centrale vacuole waar het (waarschijnlijk indirect) anthocyanen destabiliseert. In epidermale cellen van wild type bloemblaadjes, welke vacuolinos

bevatten, accumuleren GFP- en RFP-fusies van het FA eiwit in eerste instantie in vacuolinos, net als andere vacuolaire eiwitten. In de vacuolinos wordt FA-GFP (of ten minste de GFP-tag) uit de membraan vrijgemaakt om vervolgens op te hopen in het lumen van de vacuolinos. Dit zou kunnen verklaren waarom FA-GFP van de vacuolino's niet verder reist naar de centrale vacuole, zoals andere vacuolaire eiwitten doen, en waarom de FA expressie in wild type bloemblaadjes niet leidt tot afbraak van de anthocyanen in de centrale vacuole. In de bloembladepidermis van *ph4* en *ph3* mutanten ontbreken vacuolinos en kan FA(-GFP) via een directe route de vacuole bereiken, net als in bladcellen, om de afbraak van anthocyanen daar te activeren. Deze bevindingen suggereren dat vacuolinos een rol hebben als poortwachter / controlepunt en bepalen dat sommige eiwitten mogen doorreizen naar de centrale vacuole, terwijl andere eiwitten uit de buurt van de centrale vacuole worden gehouden. Deze nieuwe inzichten in de controle van bloempigmentatie (en fruitpigmentatie) via de regulatie van anthocyaan afbraak, kunnen nuttig zijn om gewasvariëteiten te genereren met nieuwe bloemkleuren of verbeterde voedingswaarde van fruit

Eerder onderzoek toonde aan dat het MBWW-transcriptiefactorcomplex essentieel is voor de vorming van vacuolinos. Waarschijnlijk stuurt MBWW de vorming van vacuolino's op een indirecte wijze, via de activering van ondergeschikte genen coderend voor eiwitten met een directe rol in het verkeer van membraanblaasjes en vorming van vacuolinos. Het mechanisme waardoor vacuolinos worden gevormd en de (MBWW-gereguleerde) genen en eiwitten die erbij betrokken zijn was echter onbekend. **Hoofdstuk 4** presenteert de identificatie van een eerste gen, RAB5a, dat door MBWW wordt gereguleerd en nodig is voor de biogenese van vacuolinos. RAB5 is een lid van de RAB-familie van kleine GTPases, waarvan er vele een cruciale rol spelen bij het specificeren van de 'identiteit' van membranen en de

binding van- en herkenning tussen blaasjes en doelmembranen. De identificatie van RAB5a, een homoloog van dierlijke RAB5 eiwitten, toonde aan dat planten-RAB5s evolutionair veel diverser zijn dan tot nu gedacht en bestaan uit drie verschillende fylogenetische groepen, die in petunia worden vertegenwoordigd door RAB5a, RAB5a1 en RAB5a2 plus de homologe (en goed bestudeerde) *Arabidopsis* eiwitten RHA1 en ARA7. De in hoofdstuk 4 gepresenteerde gegevens tonen aan dat het *RAB5a* gen wordt geactiveerd door het MBWW-complex en dat het RAB5a-eiwit nodig is om de fusie van een subpopulatie van prevacuolaire compartimenten (PVCs) te bewerkstelligen om aldus vacuolinos te vormen. Interessant is dat RAB5a1 en RAB5a2 zich in andere cellulaire compartimenten bevinden dan RAB5a, en RAB5a ook niet functioneel kunnen vervangen. Dit benadrukt de centrale rol van de RAB5a GTPase bij de vorming van vacuolinos en geeft inzicht in de diversificatie van verschillende vacuolaire compartimenten tijdens de evolutie, door duplicatie en neofunctionalisatie van genen die coderen voor belangrijke regulatoren van het intracellulaire verkeer van membraanblaasjes.

Het verlies van vacuolino's in *rab5a* mutanten en de vorming van vergrote vacuolinos door expressie van *RAB5a* vanaf de constitutieve *35S*-promoter in Petunia bloemen, gaat gepaard met veranderingen in de afmetingen en vorm van de cellen in de bloemblad epidermis. Een dergelijk effect op epidermale cellen kan de kleur van de bloembladen en zelfs de voorkeur van dierlijke bestuivers veranderen. Een verdere analyse van de effecten van RAB5a-activiteit op de celstructuur kan mogelijk additionele inzichten in de biologische functie(s) van vacuolinos verschaffen.

De zeven bekende *PH* loci in petunia zijn ontdekt via mutanten met blauw-violette bloemen in plaats van de wilde type rood-violette kleur. Deze *PH* loci zijn essentieel voor de verzuring van de centrale vacuolen in de epidermis van bloembladen en

daardoor de protonering van de anthocyaan pigmenten en het ontstaan van een rood-violette bloemkleur. Tot dusver waren zes van de zeven *PH* loci moleculair geïdentificeerd en hun biologische functies beschreven. Het *PH7* locus was tot dusver nog niet gekarakteriseerd. **Hoofdstuk 5** beschrijft de moleculaire identificatie van het *PH7* gen, middels een vergelijking van de transcriptomen van bloemblaadjes van een wild type lijn (*PH7*), een instabiele *ph7* mutant met een transposon insertie, en een stabiele *ph7* mutant. *PH7* codeert voor een monosaccharidesuiker transporter die sterk lijkt op – en vermoedelijk homolog is aan – de vacuolaire glucose/proton symporter ERD6-like6 van *Arabidopsis*. Mutaties die het *PH7* eiwit op verschillende punten afkappen resulteren in duidelijke defecten in de vacuolino-route. Afhankelijk van het punt waar *PH7* is afgekapt blokkeert ofwel de vorming van vacuolinos (en mogelijk latere stappen), dan wel het transport van vacuolinos naar de centrale vacuole. Dit laat zien dat *PH7* betrokken is bij meerdere stappen in de vacuolino-route en dat *PH7* via verschillende mechanismen en domeinen bijdraagt aan de vorming van vacuolino's en het daaropvolgende transport van vacuolinos naar de centrale vacuole.

Deze bevindingen leveren een belangrijke ondersteuning voor eerdere hypothesen dat membraan-transporters additionele functies hebben in membraan verkeer en mogelijk als structurele component fungeren in de herkenning en uiteindelijk fusie van membraanblaasjes en doelwit membranen.



## List of Publication

Faraco M.\*, Li Y.\*, **Li S.\***, Spelt C., Di Sansebastiano G.P., Reale L., Ferranti F., Verweij W., Koes R., and Quattrocchio F.M. A tonoplast P<sub>3B</sub>-ATPase mediates fusion of two types of vacuoles in petal cells. *Cell reports* **19**, 2413-2422 (2017). (\*Co-first authors)

Strazzer P., Spelt C.E., **Li S.**, Blik M., Federici C.T., Roose M.L., Koes R., and Quattrocchio F.M. Hyperacidification of *Citrus* fruits by a vacuolar proton-pumping P-ATPase complex. *Nature communications* **10**, 744 (2019).

**Li S.**, Cerri M., Strazzer P., Li Y., Spelt C.E., Blik M., Vandenbussche M., Martínez-Calvó E., Lai B., Reale L., Koes R., and Quattrocchio F.M. An ancient RAB5 governs the formation of additional vacuoles and cell shape in petunia petals. *Nature communications* (2019). (Under Revision)

**Li S.\***, Passeri V.\*, Strazzer P., Guzzo F., Spelt C.E., Vandenbussche M., Blik M., Koes R., and Quattrocchio F.M. FADING, a vacuolar metalloredutase that destabilizes anthocyanins in plant vacuoles (\*Co-first authors; in preparation)



# Acknowledgments

Before I start my PhD, nobody told me that it would take more than five years to finally get the “permission” to write an acknowledgement. What a long journey full of ups and downs. Luckily, I have not travelled alone. At this moment, it’s my great pleasure to express my sincere appreciation to all of you who supported me in the science and daily life.

First of all I would like to express my special appreciation to my promoter Prof. **Ronald Koes** and co-promoter Dr. **Francesca Quattrocchio**. Six years ago, **Ronald**, it was you who received my email of PhD application and forwarded it to Francesca providing me in this way the opportunity to join your lab and fall in love with the colorful world of *Petunia*. Whenever I have some questions about research, even basic ones, you always explain everything clearly to me with huge patience. Your deep insights and creative ideas have quite often inspired me and helped me to unravel the puzzles raised during my research. I really enjoyed the way you give talks and learned from you to make jokes to prevent audiences from falling asleep during my own talks. To write a research paper and thesis is not as easy as isolating protoplast from petunia for someone like me that is not a native English speaker. So, you spent a lot of time and effort on teaching me how to organize figures, rephrase sentences, and make your findings become an interesting story to readers. Your rigorous attitude about science and optimistic attitude about challenges have influenced me deeply and will remain a lighthouse in the dark that will guide me forward in my life. **Francesca**, as my co-promoter, you take care of me not only in research but also in my daily life. All what you have done for me makes me feel at home here. I could not imagine to manage without your help. How I could have successfully wrote my first proposal, gave my first presentation, applied my first EMBO fellowship, and accomplished my PhD thesis, and so on. I feel free when working with you and I am willing to chat with you, as your office is always open



and you can always easily understand what I am thinking of, and assist me in dealing with all kinds of issues. You encourage me when I am down, and cheer with me when I am happy, which give me lots of strength and let me never feel lonely on the journey. You are full of passion and creative ideas in the research, and have penetrating insight in transferring basic research into applied science. You taught me how to establish contacts and cooperate with groups worldwide which took me into a world of cooperation. What I've learned from you will definitely be a benefit for me throughout my life.

Second, I would like to thank **Kees**, **Bets** and **Tijs**. Dear **Kees**, the “lab father”, thank you so much for helping me to set up experiment in the lab and my life in Amsterdam. I can only try to imagine how it was difficult for you to communicate with me because of my poor spoken English at the beginning. In spite of this, each time I had some questions for you, you were always willing to give me a hand with huge patience and pleasure. Still remember that you drove me to the bank and shopping center, guided me how to go home by bike, and introduced me all kinds of tips to live in Amsterdam. You shared with me your experience and insight on research without reservation what led me to accomplish experiments much easier. Besides, your sense of humor and knowledge of China impressed me a lot and built in our lab a nice atmosphere. What a pity that you retired before I got my PhD degree! I wish you all the best and hope you and **Cora** are going to enjoy a great retirement life. Dear **Bets**, our “lab mother”, I am really grateful to you for giving me your fancy bike as a gift when I just arrived Amsterdam. For the last five years, I explored all of Amsterdam with it and regarded it as one of my “old friend”. I was shocked and deeply sad when I lost it last year. You, Bets, are such a warm hearted person. Whenever I had some issues that brought me to deal with Dutch, you are always the first person to come into my mind. Thank you very much for organizing all kinds of lab activities, as this provides me a nice platform to know our colleagues and contributes a lot of good memories to my life in Amsterdam. I really appreciated

that you keep the lab well-organized and the petunia seeds safe that made life of all of us in our lab much easier. Dear **Tijs**, the “Python master”. I really admire your passion and perseverance on doing things that you are interested in. It’s really nice to work with you. Together with you, we made a very fancy timelapse video on *ph7* mutant flower. I really appreciated the efforts and time you dedicated to write scripts to help me to successfully identify the last *PH* loci - *PH7*. What an amazing work you have done. I am also grateful that you agreed to be my paranymph.

My thanks also go **Pamela**, **Yanbang**, **Biao** and **Jihed**. Dear **Pamela**, or Salad Pamela, thank you very much for your accompany all the time. You are such a kind and nice person that made me feel free to chat with you. Whenever I was in a hard time, you were always there and you were the one who comforted me. I acknowledged and really appreciate our friendships. You taught me how to do petunia transformation and GUS staining long ago. I enjoyed your presentations each time and I learned from you how to organize the talk. Thank you so much for accepting my invitation to be my paranymph. I wish you would always have nice weather when you are on a flight. Dear **Yanbang**, the real master of protoplast, it was you who gave me a lot of information and suggestions on the application of PhD in our lab that brought me here. Thank you so much for picking up me at the airport and preparing two bags of noodle for me when I just arrived in Amsterdam. Without your help, I cannot image how I could have survived the hard time at the beginning. I am really grateful to you for guiding, supporting and encouraging me on both research and daily life. You introduced me into the world of vacuolino and taught me how to work with protoplast and confocal microscopy without any reservation, which have become the most powerful toolkit throughout my PhD career. You are a so intelligent and sharp person, I enjoyed a lot to work and discuss with you. Dear **Biao**, or pasta Biao, thanks a lot for all your valuable suggestions and assistance on my projects. Whenever I meet some problems, you were always pleased to help me to find the way out. You are knowledgeable and well-informed,

and always willing to sharing with me what you have come to know. I really appreciated your company when I was working till late and enjoyed the time with you. Dear **Jihed**, the handsome man, it was a great pleasure for me to sit beside you. I am really grateful to you for your support and encouragement when I was through a tough time. Thank you so much for spending time and efforts on making the video to make me cheer up. What an amazing video, I was so moved. I really admired your optimistic attitude to research and life, and pretty enjoyed the time with you. Wish you doing your defense soon. Good luck, handsome man!

Then I would like to express my appreciation to all the colleagues in the PDEG group who have supported me during my PhD. I would like to thank **Roeska** for guiding me in the lab and encouraging me for the first presentation, **Valentina** for assisting me with the study of the subcellular localization of FADING, **Marillis** for helping me sorting cells with the flow cytometry, **Aqibo** for sharing all kind of delicious Pakistani food, **Rechien** for your help to keep our lab well organized and make it working smoothly, **Pieter** for the help in taking care of my plants. Dear **Maïke**, thank you for all your helpful suggestions on my project during lab meeting. I really appreciated your recommendation and suggestion on finding postdoc position. Dear **Paul**, I really admire your passion on research, and thanks a lot for organizing the “lab day out” party at your place, and preparing amazing Indonesia food for us. Dear **Kai** and **Lichun**, I am sorry I did not give you enough assistance when I was busy with writing my thesis. From my side I really appreciated all of your help. I wish you all the best in making big achievement in your research. My thanks also go to **Till**, **Blaise**, **Xinyue**, **Sara**, and **Ting**, it was my pleasure to meet all of you and best wishes for the future. I would also like to thank all the students I supervised, **Andrea**, **Santhe**, **Mila**, **Jan** and **Enric**. Thank you so much for your efforts and contribution on my projects. All of you are such talent for science. I am really proud that you all have moved one step further in the field of science. Dear **Mila**, I wish you all the best with your PhD project and life. Dear **Jan**, wish you all

the best in your experiment and in enjoying life. Dear **Enric**, I wish you to make big achievements in your research and enjoy the life with **Bora**.

I would like to expand my thanks to all the other people outside our group. Dear **Lorenzo. Frigerio**, from University of Warwick, UK, thank you so much for helping me to apply for the EMBO short term fellowship and providing me an opportunity to work with you for three months. Your valuable suggestion and comments on my project helped me a lot to improve my PhD thesis. I admired your innovative ideas and deep insights in science, and benefited a lot from the discussion with you. It is also a great honor to have you as the member of my defense committee. Dear **Mistianne Feeney**, from University of Warwick, UK, I highly appreciated all your help in introducing me to all the people in the department, guiding me in the lab, designing experiments and arranging stuffs for my project, taking care of my plants, having lunch with me, and giving a lot of tips for living in Coventry. No matter how small where the questions coming from me, you were always pleased to help me to find the way out. Whenever I was struggling with the writing of thesis, you always smiled and encouraged me, which gave me a lot of strength to keep on. I pretty enjoyed those moments and your company. I wish you all the best. Dear **Remko Offringa, Dorus Gadella, Martijn Rep** and **Teun Munnik**, I really appreciated all of you accepting the invitation to be member of my defense committee and spending your time reading and commenting my thesis. Many thanks for all the suggestions and comments which helped a lot to improve it. Dear **Michiel Vandenbussche**, thanks a lot for your help by sharing transposon insertion mutant seeds. I have very much enjoyed being the host for your Green Life Science seminar at Science Park. Dear **Aureliano Bombarely**, I very much appreciated your efforts in helping me making RNA-seq library and arranging sequencing. Dear **Lara Reale** and **Martina Cerri**, thank you very much for making all kinds of amazing microscopy images. Your work made my thesis even more colorful. My special thanks go to **Michel Haring** and **Rob Schuurink** for all the

suggestion and ideas on my project during my talks in PSM and GLS seminar. I also thank **Ringo van Wijk** and **Michel de Vries** for sharing chemicals and strains, and for all kinds of help in the lab.

Dear **Walter Verweij** and **Gert-Jan de Boer**, thank you so much for providing me the opportunity to join Enza Zaden. I admired your innovative ideas on developing valuable traits for vegetable breeding. Meeting with you always increases my knowledge on breeding and I always got inspired by the scientific discussion with you to design new experiment for further researches.

Then I would like to thank all the Chinese friends. My special thanks first go to **Bai Yuechen**, thank you so much for helping and supporting me all the time. When I was a master student, you guided me in the experiment and helped me in designing them for my thesis. Without your assistance and encouragement, I cannot image how difficult it would be for me to get the CSC fellowship. Whenever I was struggling with my PhD project, you were always pleased to help me to find out the way, which gave me a lot of strength to keep on. You are such a warmhearted and intelligent person. I really admired your passion and deep insights toward the scientific research. I wish you all the best in your research and life. Dear **Li Chenglei**, I highly appreciated all your help in my research and daily life. You taught me not only how to do research but also how to forge myself in the society. What I've learned from you will be a benefit me throughout my whole life. Thank you very much for helping me arranging all kinds of documents for my PhD defense. Best wishes to you and your family. Dear **Luo Xiaopeng**, **Gao Fei** and **Yao Panfeng**, thank you so much for visiting me at Amsterdam and bringing me lots of gifts from China. I really enjoyed the time with you and best wishes to all of you. Dear **Zhang Qianqian**, I am really grateful to you for helping me to find a fancy apartment in Amsterdam. Thanks a lot for all the useful tips to work and live in Amsterdam. I wish you and **Yanbang** all the best and enjoy your life. Dear **Dong**

**Lin** and **Sun Pulv**, thanks a lot for all your help in the lab. I enjoyed the conversations with you a lot. Dear **Liu Jie**, **Xue Jieshen**, **Wang Yanting**, and **Li Changsheng**, thank you so much for all kinds of help and for all the fun during the parties together. I am grateful to all of you for your company and best wishes to all of you.

I would also like to thank Chinese Scholarship Council and University of Amsterdam for offering me the financial support and the platform for conducting experiments.

All good things come to an end. It was so lucky that I had all of you on this journey to be together with me. What I have experienced with you will become an unforgettable and precious memory in my mind, which will beneficially affect my whole life. In the end, I would like to express again my sincere gratitude to all of you – the lovely people who helped me in my life.

而立未立，惑而不惑。感父母生我养我之恩，慨外婆牵我挂我之情，谢岳父母知我信我之义。此恩此情此义，无以为报。唯望早日安生立命，以尽孝道！  
芳华易逝，真爱永藏。几多欢乐仅你与我分享，几多艰难只你与我肩扛。未有你身后默默的付出，哪来我台前的光芒。颖颖，此生遇你，幸也！余生有你，足矣！只望，执子之手，与子偕老！