Uniwersytet Rolniczy im. Hugona Kołłątaja w Krakowie

Wydział Biotechnologii i Ogrodnictwa

Kinga Kęska

Nr albumu: 999

Identyfikacja ncRNA uczestniczących w potranskrypcyjnej regulacji ekspresji genów w warunkach stresu hipoksji u ogórka (*Cucumis sativus* L.)

Autoreferat rozprawy doktorskiej

Praca wykonana pod kierunkiem

Prof. dr hab. inż. Adeli Adamus

we współpracy z promotorem pomocniczym

Dr inż. Małgorzatą Czernicką

w Katedrze Biologii Roślin i Biotechnologii

Kraków, październik 2021 rok

Uniwersytet Rolniczy im. Hugona Kołłątaja w Krakowie
Wydział Biotechnologii i Ogrodnictwa
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Pracę doktorską realizowałam w Katedrze Biologii Roślin i Biotechnologii w latach 2016-2021 w ramach studiów doktoranckich prowadzonych w Studium Doktoranckim Uniwersytetu Rolniczego im. Hugona Kołłątaja w Krakowie. Niniejsza praca została wykonana w ramach projektu badawczego finansowanego przez Ministerstwo Rolnictwa i Rozwoju Wsi pt. „Analiza wpływu hipoksji na zwiększenie tolerancji na stresy u pomidora (*Lycopersicon esculentum* L.) i ogórka (*Cucumis* sativus L.)" (HOR hn-801-PB-12/15) oraz dotacji przyznanej przez Ministerstwo Nauki i Szkolnictwa Wyższego.

W trakcie odbywania studiów doktoranckich otrzymałam Stypendium Naukowe finansowane przez Narodowe Centrum Nauki w ramach projektu pt. „Molekularne i fizjologiczne mechanizmy wpływu wanadu na pobieranie jodanów i jodosalicylanów przez sałatę oraz jej przeciwnowotworowe działanie testowane na ludzkich liniach komórkowych", czego efektem jest publikacja Smoleń i in. (2021), której jestem współautorką. W trakcie studiów doktoranckich byłam również wykonawcą badań zleconych przez firmę Intermag sp. z o.o.

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Spis treści

Wykaz publikacji stanowiących rozprawę doktorską

Publikacja nr 1:

Kołton A, **Kęska K**, Czernicka M. 2020. Selection of Tomato and Cucumber Accessions for Waterlogging Sensitivity through Morpho-Physiological Assessment at an Early Vegetative Stage. *Agronomy* 2020, *10*, 1490.
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Punktacja $MNiSW_{2020}$: **100**
IF_{2020}: **3,417**

Publikacja nr 2:

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Punktacja $MNiSW_{2020}$: **100**, autor korespondencyjny
IF_{2020}: **4,096**

Publikacja nr 3:

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https://doi.org/10.3390/ijms22158197

Punktacja $MNiSW_{2020}$: **140,** autor korespondencyjny
IF_{2020}: **5,923**

Sumaryczna punktacja MNiSW: 340

Sumaryczny IF: 13,436

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   IF_{2020}: -

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   Punktacja $MNiSW_{2020}$: 100
   IF_{2020}: 2,925

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   IF_{2020}: 4,570

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   Punktacja $MNiSW_{2020}$: 100
   IF_{2020}: 5,753

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IF_{2020}: **30,982**
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Liczba cytowań: **24**, Liczba cytowań bez autocytowań: **21**

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Wykaz używanych skrótów i terminów

lncRNA – długie niekodujące RNA (ang. *long-noncoding RNA*)

miRNA (microRNA) – małe niekodujące RNA

ncRNA – niekodujące RNA (ang. *noncoding RNA*)

1 kb – 1000 par zasad

1xH – rośliny traktowane stresem zalania systemu korzeniowego wodą przez 7 dni

2xH – rośliny traktowane siedmiodniowym stresem zalania dwukrotnie, z 14-dniowym okresem regeneracji pomiędzy traktowaniami

Rec – rośliny, które zostały poddane siedmiodniowemu stresowi zalania systemu korzeniowego wodą, a następnie przeszły w okres 14-dniowej regeneracji

DH2 (WL-T) – linia DH (ang. *double haploid line*) ogórka, wyselekcjonowana jako obiekt tolerancyjny na stres zalania

DH4 (WL-S) – linia DH (ang. *double haploid line*) ogórka, wyselekcjonowana jako obiekt wrażliwy na stres zalania

1. STRESZCZENIE

Rośliny nieprzerwanie narażone są na niekorzystne czynniki środowiskowe. W ostatnich latach zmieniające się warunki klimatyczne wpływają na występowanie nie tylko suszy, ale także nadmiernych opadów, które zalewając systemy korzeniowe upraw generują brak dostępu tlenu tzw. stres hipoksji. Rośliny muszą szybko aktywować mechanizmy obronne na poziomie morfologicznym, fizjologicznym i molekularnym, pozwalające im na przetrwanie i adaptację do warunków stresowych. Mechanizmy zachodzące na poziomie molekularnym najczęściej dotyczą zmian w poziomie ekspresji genów, która może być m.in. regulowana przez niekodujące cząsteczki RNA (ncRNA).

Celem badań była identyfikacja ncRNA u ogórka, w odpowiedzi na stres hipoksji wywołanego przez zalanie systemu korzeniowego wodą. Do ncRNA zaliczamy między innymi krótkie (miRNA) i długie (lncRNA) cząsteczki RNA. Dodatkowo, celem było sprawdzenie wpływu drugiego zalania roślin ogórka na zmiany ekspresji genów. W wyniku przeprowadzonych wstępnych analiz biometrycznych i fizjologicznych wyselekcjonowano dwa obiekty ogórka (linie podwojonych haploidów) o przeciwstawnej reakcji na stres hipoksji tj. DH2 (tolerancyjny) i DH4 (wrażliwy). Złożono transkryptomy dla obydwu obiektów ogórka oraz przeprowadzono analizę różnicowej ekspresji genów, która wykazała różnice pomiędzy badanymi obiektami poddanymi stresowi zalania. Większą pulą genów oraz lncRNA o różnicowej ekspresji względem roślin nietraktowanych stresem charakteryzowała się linia wrażliwa DH4 w porównaniu do linii tolerancyjnej DH2. Dokonano również sekwencjonowania miRNA. Zidentyfikowano nowe miRNA ulegające regulacji u ogórka pod wpływem stresu zalania. Najmniejszą liczbę miRNA o różnicowej ekspresji określono dla roślin linii DH4 zalanych jednokrotnie, a największą dla linii DH2 poddanej stresowi zalania dwukrotnie. Zidentyfikowano szlaki i procesy, w które zaangażowane są geny kodujące białka i lncRNA o różnicowej ekspresji względem roślin kontrolnych pod wpływem jednokrotnego i dwukrotnego zalania systemu korzeniowego wodą. Ponadto, określono geny docelowe potencjalnie regulowane przez miRNA w trakcie zalania. Wskazano także cząsteczki miRNA oraz lncRNA biorące udział w początkowej reakcji na stres hipoksji. Dodatkowo, zidentyfikowano 6 genów oraz 71 cząsteczek lncRNA, które potencjalnie mogą być odpowiedzialne w nabywanie tolerancji na stres zalania u ogórka. Po raz pierwszy określono interakcje, jakie mogą zachodzić pomiędzy miRNA i lncRNA w trakcie odpowiedzi na stres hipoksji, wskazując sposoby regulacji cząsteczek ncRNA między sobą.

Z wykorzystaniem technologii RNA-Seq oraz miRNA-Seq zsekwencjonowano po raz pierwszy transkryptomy oraz krótkie RNA badanych obiektów ogórka traktowanych jednokrotnie i dwukrotnie stresem hipoksji. Dane te stanowią źródło do dalszych analiz molekularnych i bioinformatycznych. Uzyskane wyniki poszerzyły wiedzę w zakresie identyfikacji cząsteczek ncRNA zaangażowanych w odpowiedź rośliny (komórki) na stres. Potwierdzono, że stres zalania może być czynnikiem stymulującym w nabywaniu tolerancji na stres hipoksji u ogórka.

2. SUMMARY

Plants are continuously exposed to unfavourable environmental factors. In recent years, changing climatic conditions have affeted not only in drought but also in excessive rainfalls, which by flooding the root systems of crops have generated a lack of oxygen, the so-called hypoxic stress. Plants must quickly activate the defence mechanisms at the morphological, physiological, and molecular levels, allowing them to survive and adapt to stress conditions. Mechanisms occurring at the molecular level most often involve changes in the level of gene expression, which can be, among other factors, regulated by non-coding RNA (ncRNA) molecules.

The aim of the study was to identify ncRNAs in cucumber in response to hypoxia stress induced by waterlogging of the root system with water. The ncRNAs include, among others, short (miRNA) and long (lncRNA) RNA molecules. Additionally, the purpose was to investigate the effect of a second flooding of cucumber plants on genes expression changes. As a result of preliminary biometric and physiological analyses, two cucumber accessions (doubled haploid lines) with opposite responses to hypoxia stress i.e. DH2 (tolerant) and DH4 (sensitive) were selected. Transcriptomes for both cucumber accessions were assembled and differential genes expression analysis was performed, which revealed differences between the waterloggied accessions. A higher number of genes and lncRNAs with differential expression relative to the non-stress-treated plants were characterized in the sensitive line DH4 compared to the tolerant line DH2. Sequencing of miRNAs was also performed. Novel miRNAs up-regulated in cucumber under waterlogging stress were identified. The lowest number of differentially expressed miRNAs was determined for plants of DH4 line flooded once and the highest for DH2 line subjected to flooding stress twice. The pathways and processes involving protein-coding genes and lncRNAs with differential expression relative to control plants under single and double waterlogging of the root system were identified. Furthermore, target genes potentially regulated by miRNAs during waterlogging were identified. The miRNA and lncRNA molecules involved in the initial response to hypoxia stress were also identified. In addition, 6 genes and 71 lncRNA molecules were identified that could potentially be responsible in the acquiring of the tolerance to waterlogging stress in cucumber. For the first time, the interactions that may occur between miRNAs and lncRNAs during the hypoxia stress response were determined, indicating the ways in which ncRNA molecules regulate each other.

Using RNA-Seq and miRNA-Seq technologies, transcriptomes and short RNAs of cucumber accessions treated once and twice with hypoxia stress were sequenced for the first

time. These data provide a source for further molecular and bioinformatic analyses. The obtained results extended the knowledge in identifying ncRNA molecules involved in the plant (cell) stress response. It was confirmed that waterlogging stress may be a stimulatory factor in the acquiring of hypoxia stress tolerance in cucumber.

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3. PRZEGLĄD LITERATURY

W ostatnich latach obserwujemy postępującą zmianę klimatu, gdzie obok występowania wysokich temperatur, problemem jest susza i nagłe zalania, powodujące ogromne straty w produkcji rolniczej. Według FAO (Organizacja Narodów Zjednoczonych do spraw Wyżywienia i Rolnictwa) światowe straty spowodowane powodziami wynoszą około 19 bilionów dolarów (http://www.fao.org/news/story/en/item/1106977/icode). Z tego powodu istnieje potrzeba szukania metod, które pozwolą na produkcję roślin tolerancyjnych nie tylko na stres suszy, ale też nadmiaru wody. Poznanie czynników molekularnych, które potencjalnie mogą być zaangażowane w reakcję roślin na stresy może przyczynić się do wyjaśnienia mechanizmów działania tych zjawisk.

Ogórek (*Cucumis sativus* L.), roślina jednoroczna reprezentująca rodzinę dyniowatych (*Cucurbitaceae*), jest trzecim na świecie najczęściej uprawianym warzywem po pomidorze i cebuli (https://www.statista.com/statistics/264065/global-production-of-vegetables-by-type). Ogórek charakteryzuje się płytkim systemem korzeniowym, przez co uważany jest za gatunek wysoce wrażliwy na działanie czynników abiotycznych, w tym na ograniczoną dostępność tlenu (Qi i in. 2012, Xu i in. 2017). Wrażliwość ogórka na nadmiar wody w glebie negatywnie wpływa na produktywność upraw (Patel i in. 2014, Liang i in. 2018).

3.1. Reakcje roślin na stres hipoksji

Zjawisko ograniczonego dostępu tlenu określone jest jako hipoksja i często występuje w środowisku naturalnym (León i in. 2021). Stres ograniczonej dostępności tlenu w strefie korzeniowej może być wywoływany u roślin w warunkach naturalnych poprzez obfite opady, które prowadzą do podtopień i powodzi, ale również w trakcie prowadzenia upraw kontrolowanych przez człowieka poprzez nieodpowiednie nawadnianie, okresowe zalewanie systemu korzeniowego pożywką w uprawach hydroponicznych czy też niedostateczną aeracją podłoża (Kläring i Zude-Sasse 2009, Vartapetian i in. 2014, Kowalska i in. 2020). Ograniczona dostępność tlenu w strefie korzeniowej wpływa negatywnie na prawidłowy metabolizm całej rośliny, hamując prawidłowy wzrost i rozwój, dlatego warunkiem ich przetrwania jest szybka aktywacja mechanizmów obronnych i adaptacja do warunków stresowych (Wang i in. 2014, Tang i in. 2021). Rośliny wytworzyły mechanizmy adaptacyjne na poziomie molekularnym, biochemicznym, fizjologicznym, które w konsekwencji prowadzą do modyfikacji morfologicznych umożliwiających transport tlenu do strefy, która jest niewystarczająco dotleniona u roślin (Licausi, 2010, Xie i in. 2021).

W literaturze znaleźć można doniesienia wskazujące, że rośliny posiadają „pamięć stresową" (ang. *stress memory*), która jest odpowiedzialna za szybszą i bardziej efektywną reakcję, gdy są one narażone na warunki stresowe po raz kolejny (Crisp i in. 2016, Galviz i in. 2020). Proces uodparniania roślin z wykorzystaniem czynnika stymulującego określony został jako priming. Traktowanie roślin czynnikiem stymulującym przygotowuje ich metabolizm na efektywniejsze działanie podczas ponownych niekorzystnych warunków środowiskowych (Martinez-Medina i in. 2016). Do czynników stymulujących tolerancję można zaliczyć m.in.: 1) związki chemiczne tj. nadtelenek wodoru (Hossain i in. 2015), wodorosiarczek sodu (Joshi i in. 2020), poliaminy (Janse i in. 2021) czy melatoninę (Savvides i in. 2016), 2) korzystne mikroorganizmy (Alagna i in. 2020) m.in. *Trichoderma harzianum* (Elkelish i in. 2020), *Metarhizium brunneum* (Cachapa i in. 2021), oraz 3) czynniki abiotyczne tj. wysoka temperatura (Jespersen, 2020, Wang i in. 2020), susza (Jin i in. 2020) czy zacienienie (Asghar i in. 2020). W przypadku badań nad ogórkiem, rośliny zostały poddane procesowi primingu przy zastosowaniu melatoniny w celu wzbudzenia tolerancji na stres wodny (Zhang i in. 2013), solny (Zhang i in. 2014) i chłodu (Posmyk i in. 2009), natomiast stosowanie szczepów *Rhizobacterium* wzbudzało tolerancję na stres suszy (Wang i in. 2012). Jednakże, wciąż nie ma danych dotyczących nabywania tolerancji na stres zalania poprzez wcześniejsze traktowanie rośliny tym samym stresem. Zjawisko primingu, czyli uodparniania roślin na niekorzystane warunki środowiskowe, jest obecnie często wykorzystywane przez hodowców do uzyskiwania odmian bardziej tolerancyjnych na warunki stresowe m.in. ze względów finansowych, gdyż jest to metoda tańsza w porównaniu do zaawansowanych technik inżynierii genetycznej (Ma i in. 2013, Nejat i in. 2018, Simopoulos i in. 2018, Betti i in. 2020, Chen i in. 2020, Johnson i Puthur, 2021).

3.2. Podział i funkcje ncRNA

W literaturze znajdujemy informacje, że morfologiczne i anatomiczne adaptacje do stresów abiotycznych i biotycznych poprzedzone są aktywacją lub represją specyficznych genów. Wykazano, że kluczową rolę regulacyjną w tych procesach odgrywają niekodujące cząsteczki RNA (ncRNA) (Barciszewska-Pacak i in. 2015, Chen i in. 2016, Sun i in. 2018). Niekodujące RNA to cząsteczki RNA, które są transkrybowane, ale jednocześnie nie wykazują zdolności do kodowania białka. Można je ogólnie sklasyfikować biorąc pod uwagę ich długość jako mikroRNA (miRNA) oraz długie niekodujące RNA (lncRNA) (Wang i in. 2017).

MicroRNA (miRNA) są najlepiej poznanymi i najliczniej występującymi w komórkach roślinnych i zwierzęcych krótkimi regulatorowymi cząsteczkami RNA o przeciętnej długości od 20 do 24 nukleotydów, których synteza przebiega w kilku etapach, głównie w jądrze komórkowym. Geny miRNA są transkrybowane głównie przez polimerazę RNA II, generując pierwotne transkrypty miRNA, zwane pri-miRNA, które następnie podlegają cięciom katalitycznym, prowadzącym do otrzymania tzw. cząsteczki pre-miRNA o długości od 50 do 100 nukleotydów. Cząsteczka ta posiada charakterystyczną strukturę drugorzędową tzw. spinki do włosów (ang. *hairpin loop, stem-loop*), która na jednym ramieniu zawiera komplementarne do siebie fragmenty sekwencji oraz pętlę z niesparowanymi nukleotydami. W kolejnym etapie dojrzałe miRNA jest wycinane z pre-miRNA i zostaje wbudowane w kompleks wyciszający RISC (ang. *RNA-Induced Silencing Complex*), który uczestniczy w procesach regulowania ekspresji genów (Stępień i in. 2017, Narjala i in. 2020). miRNA kontrolują ekspresję genów na poziomie potranskrypcyjnym na zasadzie hamowania translacji mRNA, bądź jego cięcia przez białko AGO1 wykorzystując komplementarność do sekwencji docelowej (Yu i in. 2017, Iki i in. 2018, Vakilian 2020). Od momentu zidentyfikowania pierwszej cząsteczki miRNA, tj. *lin-4* u nicienia (*Caenorhabditis elegans*) (Lee i in. 1993) odkryto setki miRNA zarówno u zwierząt, roślin, jak i wirusów, wskazując na ich regulacyjną rolę w licznych procesach biologicznych i metabolicznych (Narjala i in. 2020). U roślin miRNA regulują między innymi prawidłowe różnicowanie się tkanek, rozwój organów (korzeni, liści, łodyg i kwiatów) oraz nasion, a także rozwój systemu naczyniowego (Xie i in. 2015). Wykazano również zróżnicowaną ekspresję poszczególnych miRNA u roślin poddanych różnorodnym czynnikom stresowym takim jak: susza, zasolenie, ekstremalne temperatury, niedobór substancji odżywczych czy metale ciężkie, co wskazuje, że cząsteczki miRNA mogą być zaangażowane również w mechanizmy adaptacyjne do warunków stresowych (Barciszewska-Pacak i in. 2016, Chaudhary i in. 2021). Zidentyfikowane zostały również miRNA, które ulegają regulacji pod wpływem stresu hipoksji u rzodkiewnika (Moldovan i in. 2009), kukurydzy (Zhai i in. 2013), dzikiego pomidora (Hou i in. 2019) oraz ogórka (Xu i in. 2019). W przypadku ogórka, identyfikacja dotyczyła miRNA, które ulegają regulacji w wyniku krótkotrwałego działania stresu hipoksji tj. 2 dni. Natomiast w literaturze brak jest informacji na temat miRNA regulowanych u ogórka pod wpływem działania długotrwałego stresu zalania.

LncRNA (ang. *long non-coding RNA*) zdefiniowane są jako długie niekodujące cząsteczki RNA o długości przekraczającej 200 nukleotydów, nie wykazujące potencjału kodującego białko, podobnie jak cząsteczki miRNA. Zlokalizowane są głównie w jądrze komórkowym, we frakcjach chromatyny, ale także z mniejszą częstotliwością w cytozolu

(Nejat i Mantri, 2018). LncRNA są to cząsteczki tkankowo specyficzne o niskim poziomie ekspresji oraz słabym poziomie konserwatywności pomiędzy gatunkami (Simopoulos i in. 2018).

LncRNA zostały sklasyfikowane w zależności od lokalizacji w genomie oraz w oparciu o funkcję, jaką pełnią w komórkach. Na podstawie lokalizacji w genomie oraz orientacji jaką przyjmują względem genów można je określić jako: 1) sensowne (ang. *sense*) – lncRNA transkrybowane z tej samej nici, w tym samym kierunku co otaczające je geny, które kodują białka, 2) antysensowne (ang. *antisense*) – lncRNA traskrybowane z nici antysensownej, 3) dwukierunkowe (ang. *bidirectional*) – lncRNA ulegające ekspresji w odległości 1 kb od rejonu promotorowego, w kierunku przeciwnym do sąsiadującego genu kodującego białko, 4) intronowe (ang. *intronic*) – lncRNA zlokalizowane w intronach kodującego genu, nie nakładające się na sąsiadujące egzony oraz 5) międzygenowe (ang. *intergenic*) – lncRNA zlokalizowane pomiędzy dwoma genami kodującymi białko (Karlik i in. 2019). Z kolei ze względu na funkcje, jakie mogą pełnić w komórce lncRNA można je podzielić na cząsteczki: 1) sygnałowe (ang. *signal*) – wzmacniają bądź hamują ekspresję genów w odpowiedzi na czynnik działający na komórkę, 2) przyciągające (ang. *decoy*) – tworzą kompleksy z czynnikami transkrypcyjnymi uniemożliwiając im dostęp do chromatyny, w rezultacie inhibując proces transkrypcji, 3) naprowadzające (ang. *guide*) – dany lncRNA wiąże białka, a następnie kontroluje relokację kompleksu rybonukleoproteinowego do specyficznych miejsc w genomie oraz 4) podtrzymujące (ang. *scaffolds*) – dany lncRNA jest w stanie połączyć kilka białek w celu uformowania kompleksu rybonukleoproteinowego (Jha i in. 2020, Wang i in. 2011).

LncRNA są najmniej poznaną grupą transkryptów w genomach organizmów żywych. Identyfikacja oraz określenie funkcji poszczególnych lncRNA zostało dotychczas szeroko opisane głównie u człowieka i u zwierząt, natomiast u roślin zidentyfikowano znacznie mniej tych cząsteczek, co wskazuje na potrzebę poszerzenia badań w tym zakresie (Budak i in. 2020, Melissari i in. 2016, Petri i in. 2015, Zhang i in. 2020). Pierwsza publikacja opisująca funkcję cząsteczek lncRNA u roślin opublikowana została w 2004 roku. Określono wówczas funkcję lncRNA opisanego jako *Enod40* u lucerny, który zaangażowany jest w relokację jądrowego białka wiążącego RNA do cytoplazmy (Campalans i in. 2004). Kolejne prace wykazały, że u roślin lnRNA odgrywają kluczową rolę między innymi w procesach związanych z rozwojem roślin (Chekanova, 2015, Liu i in. 2018a, Liu i in. 2018b), ale również ulegają regulacji pod wpływem stresów abiotycznych takich jak zasolenie (Deng i in. 2018, Zhang i in. 2019, Sun i in. 2020), chłód (Li i in. 2017, Liu i in. 2018c), susza (Muthusamy i in. 2015, Ding i in.

2019, Pang i in. 2019), czy również hipoksja (Li i in. 2020, Yu i in. 2020). Dotychczas poznano potencjalną funkcję jednej cząsteczki lncRNA regulowanej w stresie hipoksji, tj. AtR8. Wykazano, że ograniczona dostępność tlenu indukuje udział At8R w mechanizmie obronnym roślin, w tym w kaskadzie z genami WRKY (Wu i in. 2012, Li i in. 2020). Jednakże, wciąż nie dokonano identyfikacji lncRNA u ogórka pod wpływem długotrwałego stresu hipoksji. Dokonano jedynie identyfikacji lncRNA zaangażowanych w odpowiedź na stres podwyższonej temperatury (He i in. 2020) oraz biorących udział w odporności na mączniaka prawdziwego ogórka (Nie i in. 2021).

3.3. Interakcje pomiędzy miRNA i lncRNA

Cząsteczki lncRNA potencjalnie mogą wpływać na regulację genów kodujących białka, ale ponadto podlegają również interakcjom z innymi niekodującymi cząsteczkami, a najczęściej z miRNA. lncRNA w interakcji z miRNA może między innymi tworzyć cząsteczkę docelową (tzw. target) regulowaną przez miRNA, jak również może ‘opłaszczać’ miRNA, uniemożliwiając jej dostęp do genu docelowego. Mechanizm ten określony został jako ‘wewnętrzne maskowanie targetu’ (ang. *endogenous target mimicry* – eTM) i ten typ interakcji jest uważany jako jeden z najważniejszych (Karakülah i in. 2016, Meng i in. 2021). Interakcje między lncRNA i miRNA odkrywają ważną rolę w regulacji wielu procesów biologicznych, w tym rozwoju, niedoborze składników odżywczych, jak również w stresach biotycznych i abiotycznych (Biswas i in. 2021). Dzięki narzędziom bioinformatycznym możliwe jest określenie potencjalnej interakcji między cząsteczkami ncRNA (Wang i in. 2017). Proces transkrypcyjnej regulacji genów jest bardzo złożony, dlatego istnieje potrzeba poszerzania wiedzy na temat zmian molekularnych, które towarzyszą reakcji roślin na stres oraz zjawisku adaptacji do warunków stresowych.

W literaturze wciąż nie znajdujemy doniesień dotyczących roli ncRNA w odpowiedzi na stres obniżonego stężenia tlenu u ogórka, co świadczy o tym, że istnieje potrzeba poszerzania wiedzy z tego zakresu. Jest to możliwe dzięki szybkiemu rozwojowi technologii głębokiego sekwencjonowania transkryptomów (RNA-Seq) i łączenia nowoczesnych metod bioinformatycznych. Wyniki uzyskane w niniejszej pracy dostarczyły informacji pozwalających na dalsze wyjaśnienie roli, jaką pełnią cząsteczki ncRNA w odpowiedzi na ograniczoną podaż tlenu u roślin.

4. HIPOTEZY BADAWCZE

W pracy doktorskiej postawiono następujące hipotezy badawcze:

1. Długie niekodujące RNA (lncRNA) zaangażowane są w regulację ekspresji genów biorących udział w odpowiedzi na stres hipoksji u ogórka.
2. Cząsteczki microRNA (miRNA) odgrywają istotną rolę w regulacji ekspresji genów indukowanych w trakcie niedoboru tlenu u ogórka poprzez wyciszanie transkryptów docelowych.
3. Niekodujące cząsteczki RNA (miRNA i lncRNA) zaangażowane są w nabywanie tolerancji na stres hipoksji u ogórka.

5. CELE BADAWCZE

Aby zweryfikować wyżej wymienione hipotezy badawcze wyznaczono następujące cele badawcze:

1. Selekcja obiektów ogórka o zróżnicowanej odpowiedzi na stres zalania. (Publikacja nr 1)
2. Identyfikacja genów oraz niekodujących cząsteczek RNA tj. miRNA i lncRNA zaangażowanych w odpowiedź na stres obniżonego poziomu tlenu u dwóch obiektów ogórka o przeciwstawnej reakcji na badany stres. (Publikacja nr 2 i 3)
3. Identyfikacja niekodujących cząsteczek RNA zaangażowanych w nabywanie tolerancji na stres hipoksji u ogórka po ponownym zainicjowaniu warunków stresowych. (Publikacja 3)
4. Wskazanie niekodujących RNA biorących udział w początkowej reakcji na stres hipoksji. (Publikacja 3)
5. Identyfikacja interakcji między cząsteczkami miRNA i lncRNA. (Publikacja 3)

Weryfikacja postawionych hipotez badawczych i realizacja wyznaczonych celów obejmowała 4 główne zadania, które kolejno realizowano.

Zadanie 1. Wyselekcjonowanie obiektów o zróżnicowanej reakcji na stres hipoksji

Wyniki przedstawiono w publikacji **nr 1** (Kołton i in., 2020).

Zadanie 2. Sekwencjonowanie RNA-Seq oraz analiza bioinformatyczna transkryptomów dwóch linii DH ogórka o różnej reakcji na stres hipoksji

Wyniki przedstawiono w publikacji **nr 2** (Kęska i in., 2021a).

Zadanie 3. Identyfikacja *in silico* lncRNA, określenie ich zróżnicowanej ekspresji w zależności od obiektu ogórka oraz traktowania stresem, a następnie walidacja lncRNA zaangażowanych we wczesną i późną odpowiedź na stres hipoksji

Wyniki przedstawiono w publikacji **nr 3** (Kęska i in., 2021b).

Zadanie 4. Sekwencjonowanie transkryptomów microRNA dwóch linii DH ogórka, identyfikacja oraz walidacja microRNA biorących udział w odpowiedzi na stres hipoksji

Wyniki opublikowano w publikacji **nr 3** (Kęska i in., 2021b).

6. MATERIAŁ i METODY

Szczegółowy opis metod badawczych, które zostały wykorzystywane do realizacji wyżej wymienionych zadań został przedstawiony w rozdziałach pt. ‘Materials and Methods’ poszczególnych publikacji odpowiednio przypisanych do zadań.

Skrócone wersje metodyki zostały opisane w streszczeniach publikacji zawartych w autoreferacie pracy doktorskiej.

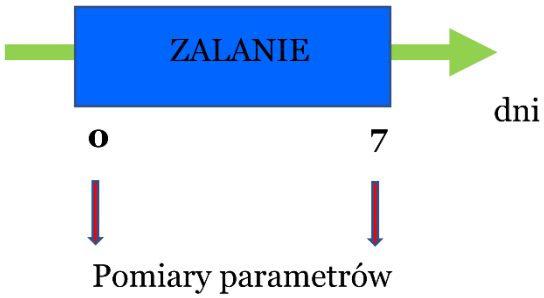
7. STRESZCZENIE ZAŁĄCZONYCH PUBLIKACJI

7.1. Publikacja nr 1

Kołton A, **Kęska K**, Czernicka M. 2020. Selection of Tomato and Cucumber Accessions for Waterlogging Sensitivity through Morpho-Physiological Assessment at an Early Vegetative Stage. *Agronomy* 2020, *10*, 1490. https://doi.org/10.3390/agronomy10101490.

Punktacja $MNiSW_{2020}$: **100**
IF_{2020}: **3,417**

Celem badań w kontekście omawianej pracy doktorskiej było wyselekcjonowanie dwóch obiektów ogórka o przeciwstawnej reakcji na stres ograniczonego dostępu tlenu w strefie korzeniowej. Materiał badawczy stanowiło 19 obiektów ogórka, których nasiona uzyskano z 3 firm hodowlano-nasiennych tj. PlantiCo Zielonki, KHiNO Polan i Spójnia HiNO Nochowo. Rośliny zostały poddane siedmiodniowemu stresowi hipoksji przez zalanie systemu korzeniowego wodą. Ocenę wpływu warunków hipoksji w obrębie systemu korzeniowego roślin na ich morfologię oraz parametry fizjologiczne dokonano po 7 dniach stresu i porównano z roślinami kontrolnymi (Ryc. 1). Ocena parametrów morfologicznych obejmowała: wysokość roślin, liczbę liści, masę części nadziemnej. Pomiar fluorescencji chlorofilu *a* pozwolił na ocenę parametrów fotosyntezy, tj. F0, Fv/F0, Fv/Fm, PI, RC/ABS oraz TFm.



Rycina 1. Schemat eksperymentu opisanego w publikacji nr 1.

W celu selekcji obiektów o przeciwstawnej reakcji na badany stres zalania wykonano analizę skupień z wykorzystaniem odległości euklidesowej. Odległości między skupieniami obliczono za pomocą metody Warda i na tej podstawie opracowano dendrogram ze skupieniami badanych obiektów. Spośród linii podwojonych haploidów, linie DH2 i DH1 zostały sklasyfikowane jako obiekty o podwyższonej tolerancji, ponieważ rośliny z grupy kontrolnej i traktowanej stresem znajdowały się w obrębie tego samego klastra (skupienia). Wybór obiektu wrażliwego na stres hipoksji opierał się na założeniu, że grupa roślin kontrolnych

(nietraktowanych) i grupa roślin traktowanych stresem będą znajdować się w oddzielnych skupieniach. Najbardziej wrażliwym obiektem okazała się być linia DH4, ponieważ rośliny kontrolne zostały włączone do klastra 1, a rośliny traktowane do klastra 2. Wskazuje to na różnicę w wartościach ocenianych parametrów pomiędzy roślinami kontrolnymi i traktowanymi stresem.

Na podstawie zróżnicowania cech morfologicznych oraz parametrów fizjologicznych pomiędzy roślinami kontrolnymi a roślinami poddanymi stresowi hipoksji dokonano wyboru dwóch obiektów ogórka, tj. DH2 i DH4, które charakteryzowały się odmienną reakcją na w/w stres. Obiekty te stanowiły materiał badawczy w następnych etapach badań realizowanych w ramach pracy doktorskiej.

7.2. Publikacja nr 2

Kęska K, Szcześniak MW, Makałowska I, Czernicka M. 2021. Long-Term Waterlogging as Factor Contributing to Hypoxia Stress Tolerance Enhancement in Cucumber: Comparative Transcriptome Analysis of Waterlogging Sensitive and Tolerant Accessions. *Genes* 12(2), 189. https://doi.org/10.3390/genes12020189

Punktacja MNiSW$_{2020}$: **100**
IF$_{2020}$: **4,096**

W niniejszej pracy przeprowadzono analizę transkryptomiczną z wykorzystaniem technologii RNA-Seq w celu zidentyfikowania genów o zróżnicowanej ekspresji pod wpływem długotrwałego zalania u dwóch obiektów ogórka o przeciwstawnej reakcji na stres hipoksji. Celem badań było także sprawdzenie jak priming tj. traktowanie przez 7 dni stresem wpływa na pamięć długotrwałą u obydwu obiektów ogórka i wskazanie potencjalnych genów zaangażowanych w nabywanie tolerancji. Dodatkowo, dokonano oceny wpływu zalania na zmiany na poziomie transkryptomu po 14-dniowym okresie regeneracji.

Materiał badawczy stanowiły rośliny dwóch obiektów ogórka o przeciwstawnej reakcji na stres hipoksji, tj. DH2 (tolerancyjny) oraz DH4 (wrażliwy). Rośliny były uprawiane w kontrolowanych warunkach do stadium trzeciego liścia (warunku uprawy zostały szczegółowo opisane w publikacji nr 2). Po 21 dniach uprawy rośliny zostały podzielone na 4 grupy doświadczalne, tj.

1) rośliny kontrolne (Ctrl), nietraktowane stresem,
2) rośliny poddane stresowi hipoksji jednokrotnie przez okres 7-miu dni (1xH),
3) rośliny, które po 7 dniach stresu były poddane okresowi 14-sto dniowej regeneracji (Rec),
4) rośliny, które zostały poddane ponownie 7-mio dniowemu stresowi hipoksji (2xH).

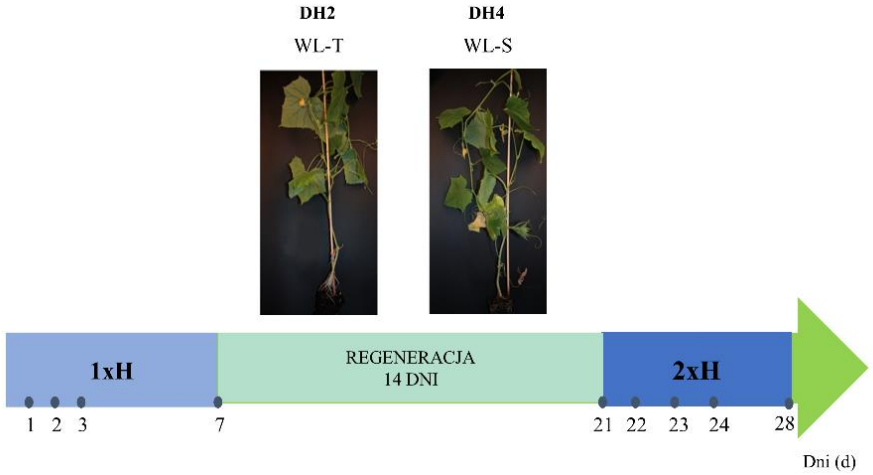
Metodyka obejmowała:

1) pomiar parametrów morfologicznych, tj. wysokość roślin, liczba liści, sucha i mokra masa łodygi oraz liści, świeża masa korzeni bocznych,
2) izolację RNA, sekwencjonowanie bibliotek RNA-Seq
3) analizę transkryptomiczną danych RNA-Seq, w tym:
   - analizę jakości odczytów oraz odfiltrowanie słabej jakości odczytów (Cutadapt ver. 1.9.1, BBMap ver. 37.02, FastQC ver. 0.11.5),
   - składanie *de novo* transkryptomów ogórka linii DH2 i DH4 (StringTie ver.1.3.3b, Cufflinks package ver.2.2.1),
   - analizę różnicowej ekspresji genów (edgeR ver. 3.20.1, DeSeq2 ver. 1.18),

- adnotację funkcjonalną (Trinotate ver. 3.1.0, TransDecoder ver. 5.0.1),
- wzbogacenie funkcjonalne genów o różnicowej ekspresji (topGO ver. 2.38.1, clusterProfiler ver. 3.6.0),
- wizualizację otrzymanych wyników (R ver. 3.6-4.0, Python ver. 3.7),

4) analizę poziomu ekspresji wybranych genów tj. dehydrogenaza alkoholowa (*adh*), oksydaza 1-aminocyklopropano-1-karboksylanowa (*aco*) oraz syntaza długołańcuchowa acylo-CoA 6 (*lacs6*) metodą qRT-PCR.

Schemat przedstawiający terminy pobierania prób do poszczególnych analiz przedstawiono na Rycinie 2.



	1	2	3	7	21	22	23	24	28
Pomiar parametrów morfologicznych				✓	✓				✓
RNA-Seq				✓	✓				✓
qRT-PCR	✓	✓	✓	✓	✓	✓	✓	✓	✓

Rycina 2. Schemat doświadczenia opisanego w publikacji nr 2. W tabeli zestawiono przeprowadzone analizy wraz z terminami, w których pobierano i analizowano próby.

W wyniku 7-mio dniowego stresu zalania systemu korzeniowego zaobserwowano wolniejszy wzrost roślin i rozwój liści, w porównaniu do roślin niezalanych, jedynie u obiektu wrażliwego na stres hipoksji – DH4. Dopiero po 14-sto dniowym okresie regeneracji odnotowano wolniejszy wzrost roślin stresowanych u obydwóch obiektów ogórka, Podobną reakcje zaobserwowano, jeżeli rośliny ponownie potraktowano stresem zalania (2xH). U obydwóch linii DH zaobserwowano rozwój korzeni bocznych (ang. *adventitious roots*) po 7-miu dniach zalania, natomiast zdecydowanie większą masę i liczbę korzeni bocznych wytworzyły rośliny linii DH2 (tolerancyjnej) w porównaniu do linii DH4 (wrażliwej).

Różnice w odpowiedzi na stres zalania u badanych obiektów ogórka zaobserwowano na poziomie transkryptomicznym. W efekcie złożenia transkryptomów *de novo* zidentyfikowano w sumie 35 712 transkryptów, które odpowiadały 18 903 unigenom. U linii

DH2 odnotowano mniejszą liczbę transkryptów (5 957) o zróżnicowanej ekspresji pod wpływem jednokrotnego stresu zalania (1xH) w porównaniu do linii DH4 (8 927). U roślin poddanych ponownie stresowi (2xH) u linii tolerancyjnej (DH2), odnotowano spadek liczby transkryptów o różnicowej regulacji w porównaniu do roślin zalanych jednokrotnie (1xH) tj. z 5 957 do 5 007. Z kolei odwrotną tendencję odnotowano u linii wrażliwej na stres (DH4), gdyż tam u roślin poddanych zalaniu po raz drugi, liczba transkryptów o różnicowej ekspresji wzrosła (11 619) w porównaniu do tych które nie były poddane ponownemu stresowi hipoksji (1xH) (8 927). Liczba genów o różnicowej ekspresji u roślin po okresie regeneracji względem roślin kontrolnych u linii wrażliwej DH4 była wyższa – 1 877 w porównaniu do linii tolerancyjnej DH2 – 654.

U obydwu linii DH ogórka, w warunkach długotrwałego stresu hipoksji (1xH), aktywne były geny związane m. in. z transkrypcją angażując podstawowe czynniki transkrypcyjne, spleicosome oraz translacją, tj. biosyntezą aminoacylo-tRNA i transportem RNA, oraz transdukcją sygnału aktywując roślinną ścieżką sygnałów MAPK. U obiektu tolerancyjnego DH2 aktywowane zostały szlaki związane z metabolizmem alaniny, asparaginianu oraz glutaminianu, podczas gdy u linii wrażliwej DH4 zaobserwowano wzrost aktywności genów biosyntezy metabolitów wtórnych.

Wskazano geny, które mogą być zaangażowane w nabywanie tolerancji na stres hipoksji u ogórka. Wśród wyselekcjonowanych były geny, których ekspresja była zwiększona zarówno u roślin zalanych jednokrotnie (1xH) u obiektu tolerancyjnego DH2 i u roślin zalanych dwukrotnie u obiektu wrażliwego DH4. Pula genów związana z nabywaniem tolerancji na stres zalania obejmowała geny kodujące: esterasę/lipasę GDSL (ang. *GDSL esterase/lipase*), aminotransferazę asparaginianu – AspAt (ang. *Aspartate aminotransferase*), dekarboksylazę glutaminianową – GAD (ang. *Glutamate decarboxylase*), syntazę sacharozy – SuSy (ang. *Sucrose synthase*), cytozolową izomerazę trizofosforanową – TPI (ang. *Triosephosphate isomerase, cytosolic*) oraz ekspansynę – EXPA (ang. *Expansin*). Geny te są kolejno związane z katabolizmem lipidów, metabolizmem aminokwasów, produkcją kwasu γ-aminomasłowego, metabolizmem sacharozy, metabolizmem glukozy oraz przebudową ściany komórkowej.

Po 14-sto dniowym okresie regeneracji zaobserwowano również różnice w ekspresji poszczególnych genów pomiędzy badanymi liniami DH ogórka. U linii DH4 (wrażliwej) odnotowano nadekspresję genów (w porównaniu do roślin nietraktowanych) związanych z procesami metabolizmu węglowodanów. Wśród nich znalazły się geny kodujące m.in. potencjalną liazę pektynianową 22 (ang. *Probable pectate lyase 22*), β-galaktozydazę 3 (ang. *β-galactosidase 3*) oraz endoglukanazę 6 (ang. *Endoglucanase 6*). Odnotowano również

zwiększoną ekspresję genów związanych z przekształceniem kwasu 1-aminocyklopropano-1-karboksylowego (ang. *1-aminocyclopropane-1-carboxylic acid (ACC)*) do etylenu, czyli oksydazy ACC 1 i oksydazy ACC 3 (ang. *ACC oxidase 1, ACC oxidase 3*) oraz genu zaangażowanego w ścieżkę przemian flawonoidów, czyli naringenina 2-oksoglutaran 3-dioksygenaza (ang. *Naringenin 2-oxoglutarate 3-dioxygenase*). Z kolei u obiektu tolerancyjnego (DH2) po 14-stu dniach regeneracji wykazano nadekspresję genu zaangażowanego w konwersję pirogronianu do acetylo-CoA, czyli kodujący dehydrogenaza pirogronianowa E1 podjednostka α (ang. *Pyruvate dehydrogenase E1 component subunit α*).

Profil ekspresji genów związanych z odpowiedzią na stres hipoksji tj. dehydrogenazy alkoholowej (*adh*), oksydazy 1-aminocyklopropano-1-karboksylanowej (*aco*) oraz syntazy długołańcuchowej acylo-CoA 6 (*lacs6*) był zróżnicowany pomiędzy badanymi liniami DH ogórka. Względny poziom ekspresji *adh* był około 2 razy wyższy u linii wrażliwej (DH4) niż u linii tolerancyjnej (DH2) po 7 dniach zalania. Wyższy poziom ekspresji u linii DH4 niż u DH2 odnotowano dla genu *aco*, gdzie u linii DH2 nadekspresja miała miejsce w początkowym etapie stresu, a następnie malała, z kolei u linii DH4 wysoki poziom ekspresji utrzymywał się przez 7 dni stresu zalania. Odwrotną zależność zaobserwowano dla genu *lacs6*, którego ekspresja była znacznie wyższa u linii DH2 niż u linii DH4.

W wyniku przeprowadzonych analiz transkryptomicznych potwierdzono zróżnicowanie w odpowiedzi na stres zalania pomiędzy liniami DH ogórka o przeciwstawnej reakcji na badany stres. Wskazano, że zalanie roślin może stanowić czynnik wspomagający nabywanie tolerancji na stres hipoksji.

7.3. Publikacja nr 3

Kęska K, Szcześniak MW, Adamus A, Czernicka M. 2021.Waterlogging-stress-responsive lncRNAs, their regulatory relatioships with miRNAs and target genes in cucumber (*Cucumis sativus* L.). *Int. J. Mol. Sci.* 22(15), 8197. https://doi.org/10.3390/ ijms22158197

Punktacja MNiSW$_{2020}$: **140**
IF$_{2020}$: **5,923**

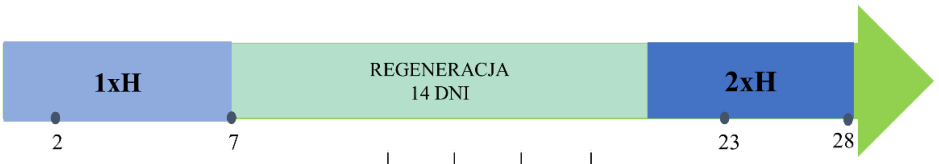
Celem badań była identyfikacja lncRNA (długich niekodujących RNA) i miRNA (krótkich niekodujących RNA) zaangażowanych w odpowiedź na długotrwały stres zalania strefy korzeniowej u dwóch obiektów ogórka o zróżnicowanej tolerancji na w/w stres. Dodatkowo podjęto próbę identyfikacji cząsteczek miRNA i lncRNA zaangażowanych w nabywanie tolerancji na ograniczoną dostępność tlenu u ogórka poprzez zastosowanie primingu, tj. zalanie systemu korzeniowego roślin. Celem było również zbadanie poziomu ekspresji wybranych ncRNA (niekodujących cząsteczek) we wczesnej fazie odpowiedzi na stres hipoksji, tj. 2 dni. Dodatkowo, zbadano interakcje pomiędzy lncRNA i miRNA w celu określenia potencjalnych szlaków regulacyjnych.

Materiał badawczy stanowiły te same grupy doświadczalne, które zostały opisane w streszczeniu publikacji nr 2. Metodyka obejmowała:

1) izolację RNA, sekwencjonowanie bibliotek miRNA-Seq,
2) analizę danych sekwencyjnych małych RNA (miRNA-Seq) z użyciem procedury CAP-miRSeq obejmującą:
   - ocenę jakości odczytów (FastQC ver. 0.10.1),
   - mapowanie do genomu referencyjnego (Bowtie ver. 0.12.7),
   - detekcję znanych i nowych miRNA (miRDeep2, ShortStack),
   - identyfikację miRNA o różnicowej ekspresji (edgeR ver. 3.20.1),
   - przewidywanie genów potencjlanie regulowanych przez zidentyfikowane miRNA (psRNATarget),
3) identyfikację lncRNA na bazie transkryptomów uzyskanych w publikacji nr 2 (Cuffcompare ver. 2.2.1 z pakietu Cufflinks),
4) wzbogacenie genów potencjalnie regulowanych przez miRNA oraz lncRNA (topGO ver. 2.38.1),
5) identyfikację interakcji między miRNA i lncRNA (psRNATarget, TAPIR, Cytoscape ver. 3.8.2),

6) analizę poziomu ekspresji:
   - 8 cząsteczek lncRNA (qRT-PCR),
   - 4 cząsteczek miRNA (TaqMan™ Small RNA Assay),
7) wizualizację otrzymanych wyników (R ver. 3.6-4.0).

Schemat doświadczenia przedstawiono na Rycinie 3.



	2	7	23	28
small RNA-Seq		✓		✓
TaqMan™ MicroRNA Assay	✓	✓	✓	✓
identyfikacja in silico lncRNA		✓		✓
walidacja lncRNA - qRT-PCR	✓	✓	✓	✓

Rycina 3. Schemat doświadczenia opisanego w publikacji nr 3. W tabeli zestawiono przeprowadzone analizy wraz z terminami, w których pobierano i analizowano próby.

Identyfikacja lncRNA została przeprowadzona z wykorzystaniem transkryptomów złożonych *de novo* (opisanych w publikacji nr 2). Całkowita liczba zidentyfikowanych lncRNA wynosiła 3 738, co stanowiło około 10 % wszystkich transkryptów określonych w transkryptomach badanych linii DH ogórka u roślin kontrolnych, traktowanych stresem zalania jednokrotnie (1xH) oraz poddanych zalaniu dwukrotnie (2xH). Analiza *in silico* pozwoliła również na klasyfikację cząsteczek lncRNA ze względu na ich lokalizację w genomie. Największy odsetek lncRNA (37%) stanowiły cząsteczki, które zlokalizowane były w obrębie egzonu genu docelowego (targetowego), ale na przeciwległej nici (ang. *Exonic overlap with the reference on the opposite strand*). Wyniki analizy różnicowej ekspresji zidentyfikowanych lncRNA w porównaniu do warunków kontrolnych wykazały, że najwięcej lncRNA regulowanych było pod wpływem jednokrotnego stresu zalania (1xH) u linii wrażliwej na stres hipoksji (DH4), tj. 1 476. Drugie zalanie systemu korzeniowego wodą (2xH) spowodowało zmniejszenie liczby lncRNA o różnicowej ekspresji u dwóch badanych linii. Porównanie puli lncRNA pomiędzy wszystkimi traktowaniami wykazało, że 303 cząsteczki były regulowane we wszystkich grupach traktowanych w porównaniu do warunków kontrolnych. Z kolei największą liczbę specyficznych cząsteczek lncRNA odnotowano u roślin zalanych jednokrotnie (1xH) u linii wrażliwej DH4 (299), a najmniejszą u roślin podwójnie potraktowanych stresem u linii tolerancyjnej DH2 tj. 9 lncRNA. Wskazano również 71

lncRNA, które potencjalnie mogą być zaangażowane w nabywanie tolerancji na stres hipoksji u ogórka poprzez wybór cząsteczek, które ulegały regulacji zarówno u roślin zalanych jednokrotnie (1xH) u linii obiektu tolerancyjnego DH2, jak i u roślin, u których zastosowano stres po raz drugi (2xH) u linii wrażliwej DH4.

Analiza bioinformatyczna wykazała, że 3 036 cząsteczki lncRNA może regulować ekspresję 2 209 genów, które znajdują się w ich najbliższym położeniu. Geny te mogą brać udział w negatywnej regulacji aktywności endopeptydazy (GO:0010951) oraz w odpowiedzi na uszkodzenia roślin (GO:0009611), a także regulować aktywność inhibitora endopeptydazy serynowej (GO:0004867). Wykazano, że lncRNA o różnicowej ekspresji u roślin niepoddanych zabiegowi primingu, zalanych jednokrotnie (1xH), linii DH2 oraz linii DH4, u których zastosowano powtórne zalanie (2xH) mogą wpływać na regulację ekspresji genów związanych z aktywnością czynnika transkrypcyjnego wiążącego DNA (GO:0003700).

Do analizy qRT-PCR wybrano losowo 8 cząsteczek lncRNA, dla których stwierdzono zróżnicowanie ekspresji pomiędzy badanymi liniami DH ogórka po pierwszym zalaniu (1xH), a także te, które zróżnicowały rośliny poddane dwukrotnemu zalaniu (2xH) od tych, u których warunki stresowe zastosowano jednokrotnie (1xH). Laboratoryjna walidacja potwierdziła zróżnicowany poziom ekspresji wybranych lncRNA po siedmiu dniach zalania (1xH) pomiędzy badanymi liniami DH ogórka, z czego 4 z badanych cząsteczek lncRNA (TCONS_00003967, TCONS_00008071, TCONS_00015763, TCONS_00019433) uległy nadekspresji u linii wrażliwej (DH4), podczas gdy u linii tolerancyjnej (DH2) zaobserwowano obniżoną ekspresję tych lncRNA. Dla 3 cząsteczek lncRNA (TCONS_00014209, TCONS_00019494, TCONS_00032986) wykazano brak wpływu stresu jednokrotnego zalania (1xH) na ich ekspresję u linii DH tolerancyjnej (brak różnicy pomiędzy traktowaniem a roślinami kontrolnymi), podczas gdy u linii DH wrażliwej ekspresja była podwyższona. Jedynie dla jednej cząsteczki lncRNA (TCONS_00021873) wykazano specyficzną, wzmocnioną ekspresję u linii tolerancyjnej po 7 dniach trwania stresu. Ponowne potraktowanie roślin stresem (2xH) spowodowało zmianę profilu ekspresji 3 cząsteczek lncRNA u linii wrażliwej i 1 cząsteczki lncRNA u linii tolerancyjnej względem kontroli. U obiektu wrażliwego, TCONS_00008071 i TCONS_00021873 uległy wzmocnionej ekspresji, z kolei TCONS_00015763 wykazał odwrotną tendencję. U obiektu tolerancyjnego ekspresja TCONS_00021873 była zwiększona po dwukrotnym zalaniu. Reakcja qRT-PCR wykazała również nadekspresję TCONS_00032986 i TCONS_00021873 po 2 dniach od indukcji stresu hipoksji tylko u linii DH tolerancyjnej (DH2), czego nie zaobserwowano u linii wrażliwej we wczesnej odpowiedzi na stres.

W wyniku sekwencjonowania 18 bibliotek miRNA zidentyfikowano 684 cząsteczki miRNA. Analiza różnicowej ekspresji zidentyfikowanych miRNA w porównaniu do roślin nietraktowanych pozwoliła zidentyfikować 19 miRNA o różnicowej ekspresji, spośród których 5 miRNA określono jako 'novel', czyli takie których sekwencje nie wykazały homologii do istniejących już w bazach, zidentyfikowanych miRNA. Ponadto wskazano specyficzne miRNA ulegające nadekspresji u roślin zalanych jednokrotnie (1xH) i dwukrotnie (2xH) u linii DH2 (tolerancyjnej) oraz roślin zalanych dwukrotnie (2xH) linii DH4 (wrażliwej), tj. csa-novel_miR4/miR169, csa-novel_miR8 i csa-novel_miR18.

Do walidacji metodą qRT-PCR wybrano 4 cząsteczki miRNA, tj. csa-novel_miR1, csa-novel_miR20, csa-novel_miR21 oraz csa-394a. Wyniki wykazały nadekspresję csa-novel_miR1 w 2. i 7. dniu trwania stresu u roślin zalanych jednokrotnie (1xH u obydwu linii DH ogórka), a z kolei u roślin ponownie poddanych stresowi (2xH) jedynie w 7. dniu stresu zalania. W przypadku csa-novel_miR20 podwyższoną ekspresję zaobserwowano jedynie po dwóch dniach trwania pierwszego zalania (1xH) u obydwu linii DH, natomiast drugie zalanie (2xH) spowodowało nadekspresję tej cząsteczki w 2. dniu od rozpoczęcia stresu tylko u obiektu wrażliwego, DH4. Nie zaobserwowano wpływu długotrwałego zalania (7 dni) na ekspresję tej cząsteczki. Z kolei, stres zalania miał wpływ na różnicową ekspresję csa-novel_miR21 jedynie u linii wrażliwej, DH4. W tym przypadku zaobserwowano zwiększoną ekspresję tej cząsteczki we wczesnym etapie odpowiedzi na stres, tj. 2 dni od rozpoczęcia stresu zarówno u roślin zalanych jedno- (1xH) i dwukrotnie (2xH).

Identyfikacja miRNA oraz lncRNA, które biorą udział w odpowiedzi na stres długotrwałego zalania pozwoliła również na określenie interakcji, jakie mogą zachodzić pomiędzy tymi cząsteczkami. lncRNA mogą stanowić cząsteczki docelowe dla miRNA, oraz mogą brać udział w mechanizmie określonym jako wewnętrzna mimikra docelowa (ang. eTM – *endogenous target mimicry*), kiedy lncRNA wiążą się z miRNA poprzez sekwencje komplementarne blokując jednocześnie interakcję między miRNA a ich autentycznymi genami docelowymi. Wykazano, że 208 lncRNA może stanowić cząsteczki docelowe dla 207 miRNA. Do dalszej analizy wybrano tylko interakcje dotyczące lncRNA, które potencjalne mogą być zaangażowane w nabywanie tolerancji na stres hipoksji u ogórka, w wyniku czego stwierdzono, że 6 z 71 cząsteczek lncRNA może być potencjalnie regulowana przez 7 cząsteczek miRNA. Cząsteczka lncRNA TCONS_00004681 może stanowić cząsteczkę docelową (targetową) aż dla trzech miRNA (csa-novel_miR341, csa-novel_miR599, csa-novel_miR600). Analiza z wykorzystaniem programu TAPIR wskazała, że 5 lncRNA może być cząsteczkami typu eTM

dla 3 miRNA, których ekspresja ulegała obniżeniu w porównaniu do roślin kontrolnych pod wpływem stresu zalania.

W wyniku przeprowadzonych analiz zidentyfikowano pulę lncRNA, które ulegają różnicowej ekspresji pod wpływem stresu zalania. Dodatkowo, wskazano cząsteczki ncRNA, które potencjalnie mogą być zaangażowane w nabywanie tolerancji na stres hipoksji. Określono interakcje, jakie mogą zachodzić między zidentyfikowanymi cząsteczkami ncRNA.

8. PODSUMOWANIE i WNIOSKI

W tabeli poniżej przedstawiono najważniejsze wyniki przeprowadzonych badań nad stresem hipoksji u ogórka.

Tabela 1. Podsumowanie najważniejszych wyników uzyskanych w ramach pracy doktorskiej ze wskazaniem różnic pomiędzy obiektami ogórka o przeciwstawnej odpowiedzi na stres hipoksji

	Badane parametry	Wariant dośw.*	Obiekt tolerancyjny DH2	Obiekt wrażliwy DH4
RNA-Seq	Liczba genów o różnicowej ekspresji względem kontroli	**1xH**	**5 957**	**8 927**
	Specyficzne szlaki KEGG wzbogacone przez geny o różnicowej ekspresji		- transport RNA - biogeneza rybosomu - biosynteza kutyny, suberyny i wosku	- rytm dobowy - metabolizm ryboflawiny
	Liczba genów o różnicowej ekspresji względem kontroli	**Rec**	**654**	**1 877**
	Specyficzne szlaki KEGG wzbogacone przez geny o różnicowej ekspresji		- ścieżka sygnału MAPK, - biosynteza karotenoidów - biosynteza fenylopropanoidu	- biosynteza aminokwasów, - interkonwersja pentozy i glukoronianu
	Liczba genów o różnicowej ekspresji względem kontroli	**2xH**	**5 007**	**11 619**
	Specyficzne szlaki KEGG wzbogacone przez geny o różnicowej ekspresji		- biosynteza zeatyny - degradacja waliny, leucyny, izolecucyny - biosynteza metabolitów wtórnych - metabolizm ryboflawiny i propionatu	- nadzór ścieżki mRNA - degradacja waliny, leucyny i izolecucyny
lncRNA	Liczba lncRNA o różnicowej ekspresji względem kontroli	**1xH**	**922**	**1 476**
	Specyficzne procesy biologiczne potencjalnie regulowane przez lncRNA o różnicowej ekspresji		- odpowiedź na stres oksydacyjny - odpowiedź na jony metali - transport anionów	- konstrukcja nukleosomu - regulacja eksportu aminokwasów - wewnątrzkomórkowy transport białek
	Liczba lncRNA o różnicowej ekspresji względem kontroli	**2xH**	**514**	**1 270**
	Specyficzne procesy biologiczne potencjalnie regulowane przez lncRNA o różnicowej ekspresji		- negatywna regulacja aktywności ATPazy - wiązanie kofaktora peptydylo pirometanowego - aktywacja aktywności kinazy białkowej	- odpowiedź na substancje nieorganiczne - regulacja lokalizacji - rozwój organów
miRNA	Liczba miRNA o różnicowej ekspresji względem kontroli	**1xH**	**6**	**5**
	Geny potencjalnie regulowane przez różnicowe cząsteczki miRNA		- czynnik odpowiedzi auksyny - podjednostka Y jądrowego czynnika transkrypcyjnego, - 2-oksydaza gibereliny - helikaza ATP zależna RNA - dekarboksylaza - S-adenozylometioniny	- czynnik odpowiedzi auksyny - białko zawierające powtórzenie pentatricopeptydowe - metionina-gamma-liaza - epimeraza/dehydrataza zależna od NAD
	Liczba miRNA o różnicowej ekspresji względem kontroli	**2xH**	**10**	**8**
	Geny potencjalnie regulowane przez różnicowe cząsteczki miRNA		- czynnik odpowiedzi auksyny - 50S rybosomalne białko L10 - 2-oksydaza gibereliny - helikaza ATP zależna RNA - dekarboksylaza S-adenozylometioniny - transporter GDP-mannozy 2 - białko SCARECROW	- czynnik odpowiedzi auksyny - białko z rodziny transporterów ABC - metionina-gamma-liaza - epimeraza/dehydrataza zależna od NAD

* 1xH – rośliny traktowane stresem zalania systemu korzeniowego wodą przez 7 dni; Rec – rośliny, które zostały poddane siedmiodniowemu stresowi zalania systemu korzeniowego wodą, a następnie przeszły w okres 14-dniowej regeneracji; 2xH – rośliny traktowane siedmiodniowym stresem zalania dwukrotnie, z 14-dniowym okresem regeneracji pomiędzy traktowaniami.

W ramach przedstawionej rozprawy doktorskiej:

1. Wyselekcjonowano dwie linie ogórka, DH2 i DH4 o przeciwstawnej odpowiedzi na stres ograniczonego dostępu tlenu poprzez zalanie systemu korzeniowego wodą. Obiekty te mogą być wykorzystane do dalszych badań związanych z poznaniem mechanizmów odpowiedzialnych za tolerancję na stres hipoksji u ogórka.
2. Złożono transkryptomy dla obydwu badanych linii DH, które zostały poddane jednokrotnemu i dwukrotnemu stresowi zalania wodą przez 7 dni. Dzięki temu została poszerzona baza transkryptów regulowanych pod wpływem badanego stresu hipoksji. Uzyskane transkryptomy stanowiły źródło danych do dalszych badań tj. do identyfikacji długich niekodujących cząsteczek RNA (lncRNA) oraz na wskazanie transkryptów, które są przez nie potencjalnie regulowane.
3. Wskazano różnice na poziomie transkryptomicznym w odpowiedzi na stres zalania pomiędzy obiektami o przeciwstawnej tolerancji na stres hipoksji. Dotyczyły one odmiennej liczby genów o różnicowej ekspresji w porównaniu z kontrolą, co umożliwiło wskazanie specyficznych szlaków regulowanych przez te geny u obiektu wrażliwego i tolerancyjnego pod wpływem ograniczonego dostępu tlenu w strefie korzeniowej.
4. Zidentyfikowano długie niekodujące cząsteczki (lncRNA) zaangażowane w odpowiedź na jednokrotny oraz dwukrotny stres zalania u obiektów ogórka wrażliwego i tolerancyjnego. Wskazano procesy biologiczne oraz funkcje molekularne, jakie pełnią geny potencjalnie regulowane przez zidentyfikowane lncRNA. Przedstawione wyniki dotyczące identyfikacji lncRNA u ogórka są pionierskimi, ponieważ dostarczyły nowych informacji o niekodujących cząsteczkach biorących udział w potranskrypcyjnej regulacji genów pod wpływem stresu hipoksji.
5. Zidentyfikowano grupę nowych miRNA ulegających regulacji pod wpływem stresu hipoksji u ogórka, które nie zostały dotąd opisane. Analiza *in silico* pozwoliła na wskazanie miRNA, które różnicowały badane linie DH pod względem odpowiedzi na stres ograniczonego dostępu tlenu. Wskazano również potencjalne geny, które mogą być potranskrypcyjnie regulowane przez zidentyfikowane miRNA poprzez m.in. wyciszanie transkryptów docelowych.
6. Ustalono interakcje, które potencjalnie mogą zachodzić między lncRNA a miRNA pod wpływem stresu ograniczonego dostępu tlenu u ogórka. Jest to pierwsze doniesienie naukowe w obszarze mechanizmów zachodzących pomiędzy ncRNA u tego gatunku.

Sukcesem było wskazanie, że lncRNA mogą stanowić cząsteczki docelowe (targetowe) do regulacji poprzez miRNA lub mogą konkurować z miRNA w dostępie do tego samego genu, wtedy lncRNA łączy się z miRNA ograniczając jego dostęp do genu docelowego.

7. Przeprowadzono walidację laboratoryjną wybranych miRNA i lncRNA, która pozwoliła na wskazanie cząsteczek regulowanych we wczesnym etapie odpowiedzi na stres, tj. po 2 dniach trwania stresu.

8. Zastosowanie primingu umożliwiło zidentyfikowanie u ogórka 6 genów i 71 cząsteczek lncRNA, które potencjalnie są odpowiedzialne za nabywanie tolerancji na stres hipoksji. Geny te związane są z katabolizmem lipidów, metabolizmem aminokwasów, sacharozy i glukozy, produkcją kwasu γ-aminomasłowego, oraz przebudową ściany komórkowej. Jest to, według mojej wiedzy, pierwsze doniesienie naukowe, które wykorzystuje zalanie roślin ogórka jako czynnik stymulujący tolerancję na brak dostępu tlenu do systemu korzeniowego oraz wskazuje konkretne obszary w transkryptomie zaangażowane w ten proces.

9. Otrzymane kompletne transkryptomy roślin kontrolnych ogórka oraz traktowanych stresem zalania systemu korzeniowego wodą stanowią źródło danych do dalszych badań związanych m.in. z identyfikacją wariantów transkrypcyjnych oraz mechanizmów związanych z ich powstawaniem.

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10. Wydruki publikacji wchodzących w skład rozprawy doktorskiej

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Selection of Tomato and Cucumber Accessions for Waterlogging Sensitivity through Morpho-Physiological Assessment at an Early Vegetative Stage

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Abstract: Waterlogging anomalies have recently increased, causing a reduction in yield and the loss of billions of dollars. Plant selection for increased tolerance to stress factors requires parameters with high sensitivity, as well as fast and inexpensive measurements. The aim of this study was to select tomato and cucumber accessions that reveal sensitivity and tolerance to waterlogging stress at an early vegetative stage. The selection of effective criteria for assessing plants was also an important issue. A total of 19 cucumber (including four highly homozygous) and 16 tomato accessions were evaluated, and plants with three true leaves were examined. The root zone of stressed plants was waterlogged for 7 days in a deep container. Morphological and physiological characteristics were obtained after 7 days of treatment and used for cluster analysis for discrimination of tolerant and sensitive accessions. Significant decreases in Fv/F_0, Fv/Fm, Area, PI ABS, ET_0/ABS, and ET_0/TR_0 parameters, as well as increases in DI_0/RC, were observed in sensitive accessions, with no changes in tolerant plants. The OJIP test parameters (Fv/F_0, PI ABS, DI_0/RC, and Area) were more sensitive in selecting for waterlogging stress than Fv/Fm. The present research can be used in breeding programs. Selected accessions will support a detailed explanation of the physiological differences in response to waterlogging stress in tomato and cucumber plants.

Keywords: submergence; OJIP test; selection criteria; hypoxia; tolerance

1. Introduction

As a result of climate change, waterlogging events have increased, causing billions of dollars' worth of crop losses [1–3]. The yield loss caused by waterlogging may vary between 15% and 80%, depending on the species (cotton, maize, wheat, rice, and soybean); soil type; and the duration of stress [4,5]. A reduction in the yield of vegetables due to flooding stress has also been observed, in tomato (*Solanum lycopersicum* L.) by 40% and sweet potato (*Ipomea batatas* L. Poir) up to 56% [6]. Understanding the morphological, physiological, and molecular mechanisms that underlie waterlogging (WL) tolerance presents a challenge to research. The establishment of selection criteria for WL-tolerant genotypes and breeding of WL-tolerant cultivars are critical for the expansion of cultivation, particularly in areas with frequent and high rainfall. An ideal WL-tolerant cultivar should not only survive waterlogging, but also rapidly recover to the control level [4].

In agricultural soils, waterlogging often occurs because of heavy rainfall, but can also be due to inadequate soil drainage. Taking into account the height of the water surface produced, flooding could be classified as waterlogging when it covers only the roots, or as submergence when water

completely covers the plant [7]. The saturation of soil with water reduces gas exchange with the atmosphere, causing the oxygen concentration to decrease rapidly and leading to O_2 deficiency (hypoxia) or O_2 absence (anoxia). Oxygen diffuses about 10,000 times slower in water than in the air, and this restricts aerobic respiration by the roots [8]. Limited oxygen availability for plants often occurs in hydroponic cultivation in environments without appropriate aeration [9] and also is induced by improper irrigation [10].

Plants growing in waterlogged soils can tolerate oxygen deficiency by shifting from aerobic to anaerobic respiration, although the latter is less efficient for ATP production. Moreover, this process produces harmful metabolic products that could cause plant death, i.e., acetaldehyde or lactic acid [11,12]. Most crops are WL-sensitive; however, the extent of damage depends on the species, the stage of development, and the climatic conditions, as well as on the duration of exposure to stress [1]. Tomato (*Solanum lycopersicum* L.) and cucumber (*Cucumis sativus* L.) are classified as sensitive to root hypoxia [13–18], although differences amongst genotypes regarding their tolerance to this stress have been reported [19–21]. The variability of responses to root hypoxia among genotypes suggests that different strategies have evolved to deal with the stress. Tolerance to waterlogging mostly depends on the ability to develop specialized structures, allowing aeration of the tissues, which includes the formation of aerenchyma, adventitious roots, stem hypertrophic lenticels, and stem cracks [22–25]. Waterlogging stress induces senescence, resulting in leaf chlorosis, necrosis, and leaf loss [26]. The root system is strongly affected, as evidenced by the reduction in wheat (*Triticum aestivum* L.) root biomass [27]. Under waterlogging conditions, physiological disturbances are induced in plants, such as stomata closure and reductions in transpiration and photosynthetic rates, leaf water potential and transport of carbohydrates, reduced absorption of nutrients, and hormonal changes [28–30].

High sensitivity parameters are used for the selection of plants with increased tolerance to a stress factor. These also need to be fast and inexpensive because analysis of huge plant populations is required. Visual symptoms (visual assessment) parameters related to agronomic characteristics, such as yield or growth, molecular markers, and physiological parameters, are used to assess plant tolerance to stress factors [31–34]. As early as 1983, researchers used chlorophyll fluorescence to assess the effect of stress on the photosynthetic apparatus of plants, with the authors of the study suggesting their usefulness in plant breeding [35]. Since then, chlorophyll fluorescence has been used in many studies to assess the effect of stress (including waterlogging stress) on green parts of plants, and these have confirmed the usefulness of this method (for example [36–38]). Therefore, in our research, we have applied growth parameters as well as chlorophyll fluorescence parameters for the evaluation of plant tolerance to waterlogging stress.

Plenty of studies have demonstrated that plants at the seedling stage were consistently used for, among others, screening genotypes that displayed tolerance to variety of stresses, such as flooding in barley (*Hordeum vulgare* L.) [37] and hypoxia in cotton (*Gossypium hirsutum* L.) [39]. In the case of the tomato, plants at the seedling stage were used for the selection of plants tolerant to chilling [40], heat [41], and salinity [42,43], whereas cucumber seedlings, according to the literature, were subjected to submergence in order to select ones tolerant and sensitive to a lack of oxygen [44]. As Zou [34] reported, plants of *Brassica napus* L. that reveal tolerance to waterlogging at the seedling stage can demonstrate the same tolerance at later developmental stages, and moreover, assessment of tolerance at the seedling stage can be more efficient.

The aim of this study was to evaluate height, weight, leaf number, and chlorophyll fluorescence parameters of tomato and cucumber accessions and their responses to waterlogging stress at an early vegetative stage. Our research could be useful in indicating accessions that may be exploited as potential parental lines in breeding programs to develop waterlogging-tolerant cultivars. Moreover, an important issue is indicating effective selection criteria for the assessment of plant waterlogging stress tolerance. The hypothesis of this study was that accessions differ with tolerance to waterlogging stress. Furthermore, their morphological and physiological characteristics can be used for discrimination of

the tolerance of tomato and cucumber plants to waterlogging stress at the seedling stage, with cluster analysis being useful for the indication of more tolerant and sensitive accessions. The presented results have the potential to be further used by breeders and scientists for developing cultivars with improved hypoxia tolerance and increased yield production under waterlogging stress.

2. Materials and Methods

2.1. Plant Materials and Cultivation

Seeds of 19 cucumber and 16 tomato accessions were provided by Polish breeding companies, i.e., KHiNO Polan, PlantiCo, and Spójnia HiNO (Table 1). Seeds were sown in 40-cell multi-pots; each cell had volume of 0.23 dm^3. Cells were fulfilled with peat substrate Klasmann KTS-2 (Germany). According to the manufacturer, the peat substrate contained, as follows (in mg dm^{-3}): 250–500 N, 170–230 P_2O_5, 320–500 K_2O, and 80–120 Mg. The salinity and pH were 2.0 g dm^{-3} and 5.5–6.5, respectively. Seeds were cultivated in a greenhouse and, after germination, were lit with supplementary radiation (High-Pressure Sodium HPS lamps) to prolong the day length to 16 h. Minimum photosynthetic photon flux density (PPFD) on plant level during the day was 80 ± 20 µmol m^{-2} s^{-1} (when only radiation from HPS lamps reached the plants). The ambient temperature during tomato cultivation was 25.1 ± 4.8 °C in the day, and 22.3 ± 6.0 °C in the night. During cucumber cultivation, the daily average temperature was 27.6 ± 6.1 °C, and the night temperature was 24.1 ± 7.1 °C.

Table 1. Description of plant material used in the study.

Cucumis sativus L.		*Solanum lycopersicum* L.		Origin
Accession	**Breeding Status**	**Accession**	**Breeding Status**	
GROT	F1 cultivar	POL 1/15	F1 cultivar	KHiNO Polan, PL
MARKUS	F1 cultivar	POL 2/15	Breeding line BC	
TYTUS	F1 cultivar	POL 3/15	Breeding line	
B1F1	Parthenocarpic cultivar	POL 4/15	Breeding line	
B2F1	Parthenocarpic cultivar	POL 5/15	Breeding line	
DH1	Double haploid line	POL 6/15	Breeding line	
DH2	Double haploid line	POL 7/15	Breeding line BC	
DH3	Double haploid line	POL 8/15	Breeding line	
DH4	Double haploid line			
PGZ-1	Breeding line	PZ 115	Cultivar	PlantiCo, PL
GMG-30	Breeding line	PZ 215	Cultivar	
GM-50	Breeding line	PZ 315	F1 cultivar	
G404	F1 cultivar	PZ 415	Cultivar	
G598	F1 cultivar	PZ 515	F1 cultivar	
		PZ 615	Cultivar	
		PZ 715	Cultivar	
		PZ 815	Cultivar	
NOE1	F1 cultivar			Spójnia HiNO
NOE2	F1 cultivar			
NOE3	F1 cultivar			
NOE4	F1 cultivar			
NOE5	F1 cultivar			

2.2. Stress Treatment

Tomato and cucumber seedlings, at the 3–4 fully expanded mature leaf stage, were divided into 2 equal groups: the Control and Stress groups. Before stress treatment, the percent volumetric water content (VWC) was measured using a Delta-T Devices SM150 soil moisture sensor kit (Delta-T Devices Ltd., Cambridge, United Kingdom) and plants were watered to obtain a soil moisture level up to 30%. The root zone of tomato and cucumber plants from the Stress group were waterlogged for 7 days (Figure 1) in a deep tray containing water. Plants from the Control group were watered as needed. The oxygen level in the air and in the water were monitored during the experiment by

a Dissolved Oxygen (DO) Meter (HI 2040-02 edge, Hanna instruments, Woonsocket, RI, USA). The oxygen concentration in the water reached a value of 2.6 mg dm^{-3} (air saturation = 29.2%, temperature = 20 °C) and that level was maintained to the end of the stress treatment, whereas in the air the oxygen concentration was 9.20 mg dm^{-3}.

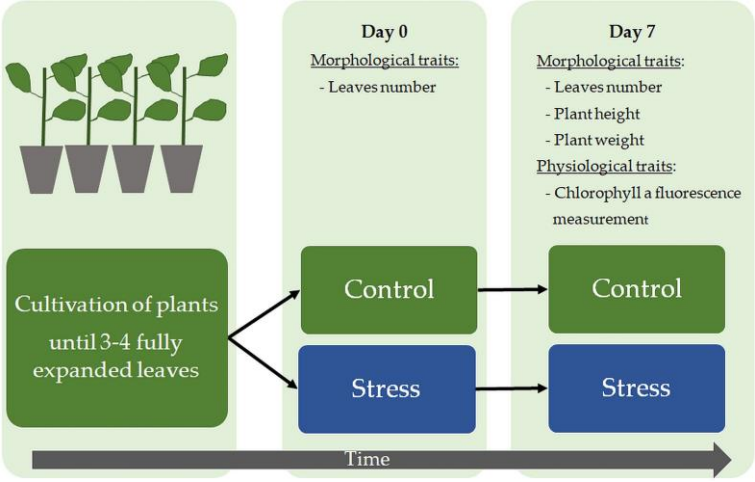

Figure 1. Scheme presenting the parameters measured during the experiment.

2.3. Growth Analysis

Before the waterlogging treatment, we labelled 20 random plants from the Control (C) and Stress groups (S) for further analysis. At the 0 time-point and after 7 days of waterlogging, we determined the numbers of leaves on Control and Stress plants. After 7days of treatment, the following parameters were measured: plant height (only shoots) (cm), measured with a ruler, and stem weight (with leaves) (g), determined by a laboratory scale (Ohaus, Parsippany, NJ, USA) (Figure 1). Plant height and weight were presented as a percentage ratio (%), assuming Control values as 100%.

2.4. Chlorophyll a Fluorescence Analysis

Chlorophyll *a* fluorescence was measured on the third leaf from the top of the plant, after 30 min dark adaptation with a special clip. The analyses were made after treatment with 3500 µmol m^{-2} s^{-1} light intensity. In the case of each accession, we performed the measurements on 8 plants from the Control or Stress groups. Chlorophyll *a* fluorescence was measured using a HandyPea portable fluorometer (Hansatech, King's Lynn, UK). The fast phase of the fluorescence transient was denoted as OJIP, where the letters indicate characteristic points on the fluorescence induction curve: O is for origin (first measured minimal level), J and I are intermediates, and P is the maximum level of fluorescence curve. For simplicity, the analysis of OJIP fluorescence transient was called the JIP-test [45]. Some of the JIP-test parameters were calculated with formulas from Stirbet and Govindjee [45] and Stirbet et al. [46], as follows: F_0 (minimum chlorophyll *a* fluorescence), Fm (maximum chlorophyll *a* fluorescence after dark adaptation), Fv (maximum variable fluorescence), Fv/F_0 (ratio of the photochemical and non-photochemical processes in photosystem II (PSII), the maximum efficiency of the photochemical processes of PSII), Fv/Fm (the maximum quantum yield of PSII photochemistry), Tfm (time to reach the maximum chlorophyll fluorescence), Area (area above the OJIP transient and Fm line), Fm/F_0 (the stable parameter in healthy leaves, value between 4–5), PI ABS (performance index on an absorption basis), ABS/RC (absorbed photon flux per PSII reaction center (RC) or apparent antenna size of an active PSII), TR_0/RC (maximum trapped exciton flux per active PSII), ET_0/RC (the flux of electrons transferred from the primary electron acceptor (QA) per active PSII reaction center), DI_0/RC (the flux of energy dissipated in processes other than trapping per active PSII reaction center), ET_0/ABS (quantum yield of

electron transport from QA), and ET_0/TR_0 (efficiency with which a PSII trapped electron is transferred from QA).

2.5. Statistical Analysis

Euclidean distances were computed using Ward's method between samples from Control and Stress groups of each tomato and cucumber accession. Prior to analysis, we standardized the data. Ward's method was applied and observations with high values of measured features were clustered together and the same rule was applied with low values of parameter observations. After cluster analysis was performed, we created a dendrogram with a marked cut-off point dividing the analyzed objects into distinct clusters. The cut-off point was set at a clear clustering point and is marked with a colored line in the graph. Differences between clusters and between Control and Stress groups were determined using Student's *t*-test. The level of significance was established as $p < 0.05$. All data analyses were made using STATISTICA 13 (TIBCO Software Inc. (2017) from Statistica (data analysis software system, version 13. http://statistica.io)).

3. Results

The presented results compared the response of 15 cucumber and 16 tomato accessions to waterlogging stress. For clear presentation, we divided the results into two subsections. Additional analyses were made with four homozygous accessions of cucumber and are presented in the Supplementary Materials Section.

3.1. Cucumber

Cluster analysis based on all tested parameters and 30 treatments (15 cucumber accessions each as Control and Stress) were classified into two discrete groups with an Euclidean distance of 20 (Figure 2). Nine treatments were included in cluster 1 and 21 others were included in cluster 2.

The differences between cluster 1 and 2 are presented in Table 2. Cluster 1 consisted of groups with favorable values of determined parameters, in contrast to cluster 2, where groups with less favorable values were clustered. Interestingly, the mean value of two parameters (increase in leaf number and Tfm) were similar in both clusters. Other parameters were significantly different between clusters. A significant decrease in growth, Fm, Fv, Fv/F_0, Fv/Fm, Area, Fm/F_0, PI ABS, ET_0/RC, ET_0/ABS, and ET_0/TR_0 parameters were observed in cluster 2 as compared to cluster 1. As well as an increase in weight, we also observed increases in F_0, ABS/RC, TR_0/RC, and DI_0/RC.

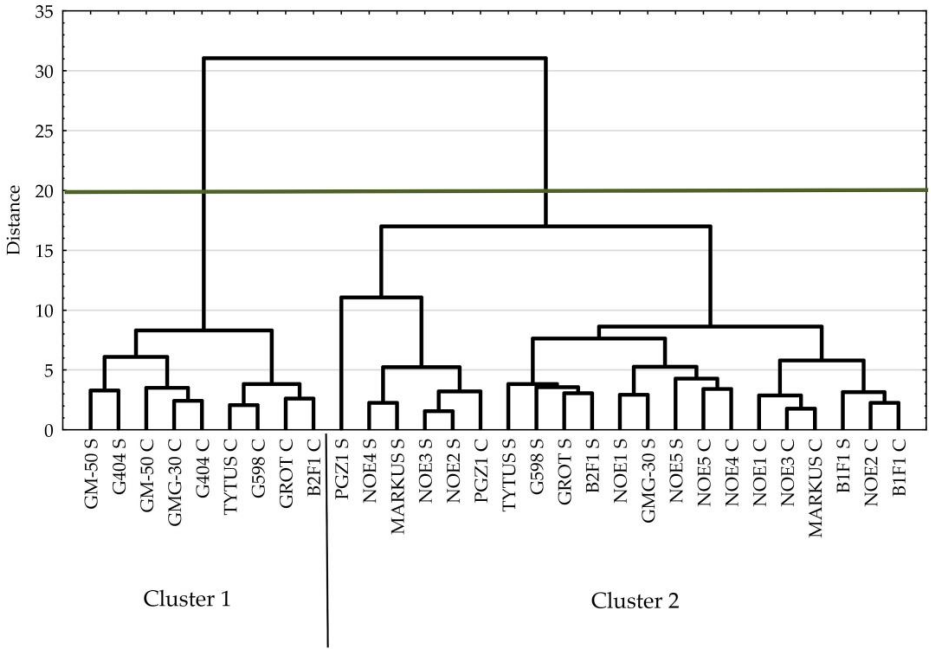

Figure 2. Results of cluster analysis for cucumber accessions using the Euclidean distance on the basis of morphological and physiological traits (Ward's hierarchical algorithm); green line indicates the cut-off point.

Table 2. Mean value of each parameter determined for both clusters (cucumber plants). Bold *p*-values indicate statistically significant differences between cluster 1 and cluster 2, estimated with Student's *t*-test and $p < 0.05$.

Parameter	Cluster 1	Cluster 2	*p*-Value
% weight change	97	103	**0.0000**
% height change	93	88	**0.0136**
Relative leaf number	1.11	1.05	0.1955
F_0	444	464	**0.0000**
Fm	2496	2397	**0.0000**
Fv	2052	1932	**0.0000**
Fv/F_0	4.65	4.22	**0.0000**
Fv/Fm	0.82	0.80	**0.0000**
Tfm	172	171	0.8759
Area	27,039	22,302	**0.0000**
Fm/F_0	5.65	5.22	**0.0000**
PI ABS	1.11	0.79	**0.0000**
ABS/RC	3.29	3.54	**0.0000**
TR_0/RC	2.71	2.84	**0.0000**
ET_0/RC	1.15	1.07	**0.0000**
DI_0/RC	0.59	0.70	**0.0000**
ET_0/ABS	0.35	0.31	**0.0000**
ET_0/TR_0	0.43	0.38	**0.0000**

On the basis of Figures 2 and 3, we assigned accessions GM-50 and G404 of both Control and Stress groups in cluster 1, which were close to each other. This meant that their parameters did not change under stress conditions, and thus these accessions could have been considered as tolerant to hypoxia stress. To select one of these, we conducted a comparison of morphological and physiological parameters in Control and Stress groups, followed by statistical analysis (*t*-test, $p < 0.05$) (Table 3). As a result, in GM-50, two morphological parameters were changed between control and stressed

conditions (the decrease of weight change and height change in stress treatment was observed), whereas in G404, four parameters were statistically disparate (the decrease of weight change, height change, and leaf number, as well as increase of Tfm in stress-treated plants were noticed). According to that analysis, we selected plants from cucumber accession GM-50 as they were more tolerant to oxygen deprivation in the root zone.

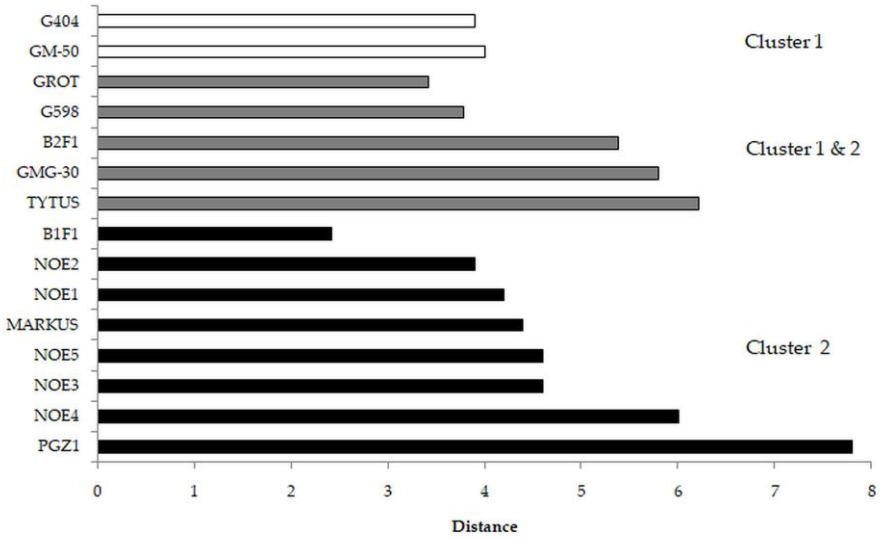

Figure 3. Euclidean distance between Control and Stress groups of each cucumber accession. White bars indicate accessions from control and stress treatments that were classified into cluster 1; light grey bars indicate accessions from control and stress treatments that were classified into different clusters; black bars indicate accessions from control and stress treatments that were classified into cluster 2.

Table 3. Mean value of each parameter determined in two cucumber accessions considered as more tolerant to waterlogging. Bold *p*-values indicate statistically significant differences between Control and Stress plants of each accession separately estimated with Student's *t*-test and $p < 0.05$.

Parameter	GM-50 Control	GM-50 Stress	*p*-Value	G404 Control	G404 Stress	*p*-Value
% weight change	100	85	**0.0000**	100	93	**0.0244**
% height change	100	67	**0.0000**	100	65	**0.0000**
Relative leaf number	1.05	1.33	0.1529	1.30	0.78	**0.0010**
F_0	423	439	0.0907	437	464	0.0696
Fm	2484	2569	0.1993	2453	2521	0.2838
Fv	2060	2130	0.2541	2016	2057	0.5182
Fv/F_0	4.88	4.85	0.8237	4.66	4.46	0.3244
Fv/Fm	0.83	0.83	0.7230	0.82	0.82	0.3969
Tfm	176	179	0.7788	164	186	**0.0351**
Area	29,868	29,264	0.6359	27,051	26,648	0.7991
Fm/F_0	5.88	5.85	0.8226	5.66	5.46	0.3243
PI ABS	1.13	1.21	0.3860	1.26	1.10	0.2351
ABS/RC	3.40	3.31	0.2075	3.24	3.37	0.2383
TR_0/RC	2.82	2.74	0.1317	2.66	2.74	0.2851
ET_0/RC	1.23	1.21	0.5859	1.20	1.21	0.7823
DI_0/RC	0.58	0.57	0.6829	0.58	0.63	0.2378
ET_0/ABS	0.36	0.37	0.8337	0.37	0.36	0.4029
ET_0/TR_0	0.44	0.44	0.8227	0.45	0.44	0.4775

The selection of sensitive cucumber accession was based on the analysis of distances between accessions and their Control and Stress groups presented in Figures 2 and 3. Accessions GMG-30 and TYTUS were selected as hypothetically sensitive accessions since their Control groups were assigned to cluster 1, whereas the Stress groups were assigned to cluster 2, indicating dissimilarity in parameter

values. TYTUS was chosen as a sensitive cucumber accession due to the number of changed parameters between control and stress conditions, i.e., 13, whereas in GMG-30, only four appeared to be different (Table 4).

Table 4. Mean value of each parameter determined in two cucumber accessions considered as more sensitive to waterlogging. Bold *p*-values indicate statistically significant differences between Control and Stress plants of each accession, separately estimated with Student's *t*-test and $p < 0.05$.

Parameter	GMG-30 Control	GMG-30 Stress	*p*-Value	TYTUS Control	TYTUS Stress	*p*-Value
% weight change	100	100	0.5298	100	99	0.8431
% height change	100	72	**0.0000**	100	62	**0.0000**
Relative leaf number	0.86	1.23	0.0838	1.52	1.05	**0.0082**
F_0	413	456	0.0605	450	469	0.1380
Fm	2441	2405	0.6090	2527	2371	**0.0049**
Fv	2029	1949	0.3058	2077	1902	**0.0023**
Fv/F_0	4.93	4.42	0.0550	4.63	4.10	**0.0057**
Fv/Fm	0.83	0.81	0.0820	0.82	0.80	**0.0035**
Tfm	173	159	0.2001	172	179	0.4821
Area	26,474	24,396	0.1341	27,277	22,771	**0.0044**
Fm/F_0	5.93	5.42	0.0549	5.63	5.10	**0.0057**
PI ABS	1.30	0.99	**0.0357**	1.04	0.75	**0.0323**
ABS/RC	3.24	3.51	0.0599	3.28	3.41	0.0802
TR_0/RC	2.69	2.82	0.1082	2.69	2.73	0.4889
ET_0/RC	1.21	1.17	0.1676	1.11	0.97	**0.0139**
DI_0/RC	0.55	0.69	0.0734	0.58	0.68	**0.0052**
ET_0/ABS	0.38	0.34	**0.0247**	0.34	0.29	**0.0161**
ET_0/TR_0	0.45	0.42	**0.0286**	0.42	0.36	**0.0200**

The parameters of both the more tolerant (GM-50) and more sensitive (TYTUS) accessions are presented in Figure 4. Control parameters were set as 1 and the parameters of Stress-treated plants were expressed as a percentage of Control. On the presented radar graph, it is easy to notice differences in the response of both genotypes to the given stress.

In the case of cucumber accessions provided by the Polish breeding company, we carried out additional experiments with highly homozygous plants (see the Supplementary Materials Section). Homozygous lines of cucumber plants were classified into two groups according to cluster analysis (Figure S1). Five treatments were included in cluster 1, and three others into cluster two. Cluster 1 consisted of groups with better values of determined parameters, in contrast to cluster 2, where groups with worse values were clustered. Differences between cluster 1 and 2 are presented in Table S1; according to Figures S1 and S2, DH2 and DH1 accessions were classified as more tolerant. As a result, DH2 was chosen as a more tolerant form for further investigation. The selection of accessions sensitive to hypoxia stress was based on the assumption that Control and Stress will be in separate clusters. The most sensitive accession was DH4, because the Control plants were included in Cluster 1 and the Stress plants in Cluster 2. This indicated a significant deterioration of parameters after stress treatment.

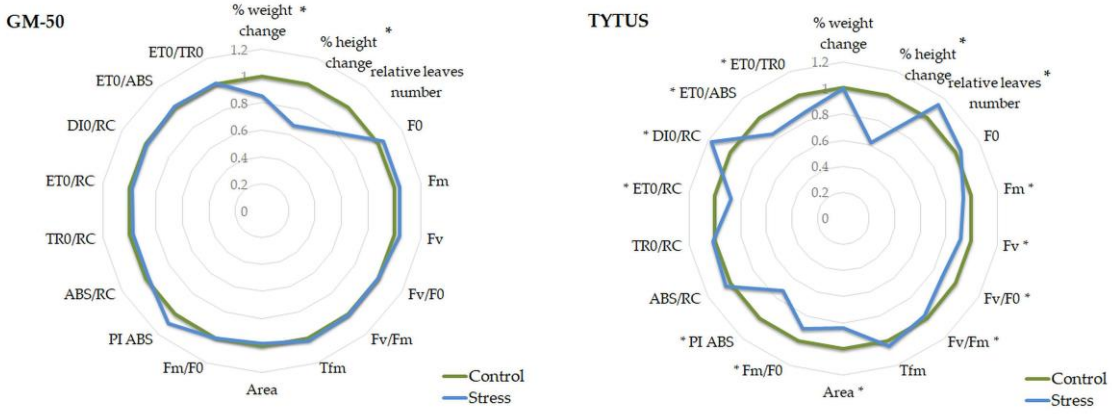

Figure 4. Radar charts comparing 18 traits estimated in Control and Stress plants of two cucumber accessions, GM-50 and TYTUS. Parameters from the Control group were set as 1 and parameters of the Stress-treated plants were expressed in relation to the Control. Asterisks indicate significant differences between Control and Stress according to Student's *t*-test and $p < 0.05$, calculated separately for each parameter.

3.2. Tomato

The Figure 5 illustrates the relationship among tomato accessions on the basis of differences in morphological and physiological parameters. It was observed that tomato plants were grouped into two main clusters with an Euclidean distance of 25 (Figure 5). In cluster one, we included 13 treatments, whereas 19 were included for cluster two.

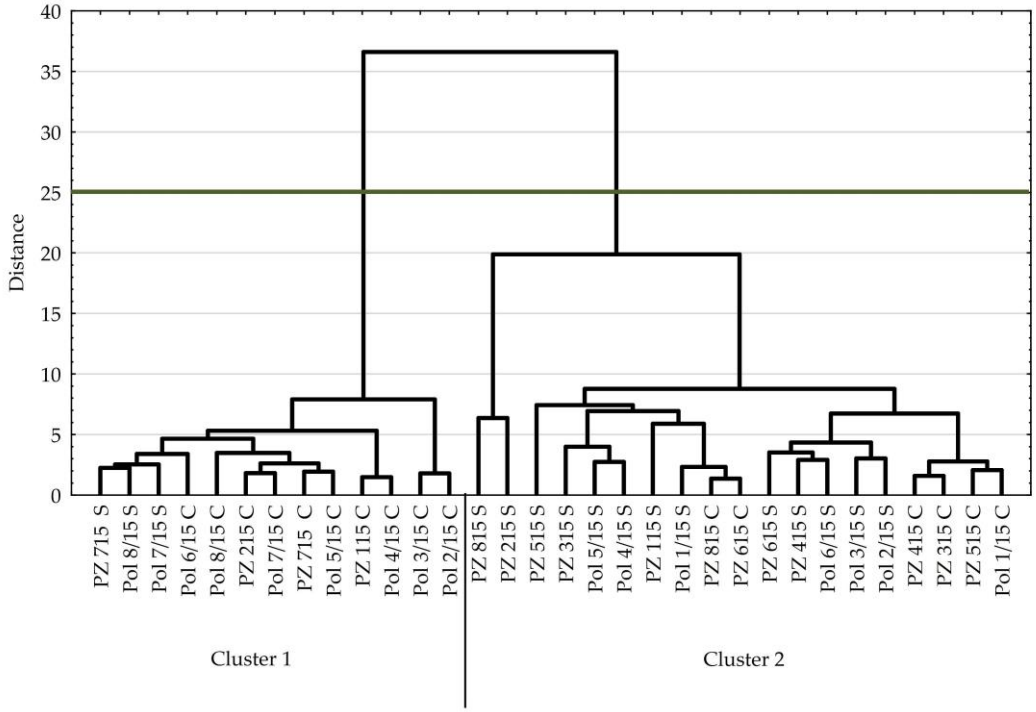

Figure 5. Results of cluster analysis for tomato accessions using the Euclidean distance on the basis of morphological and physiological traits (Ward's hierarchical algorithm); green line indicates the cut-off point.

The differences between cluster 1 and 2 are presented in Table 5. Cluster 1 consisted of groups with favorable values of determined parameters, in contrast to cluster 2, where groups with worse values

were included. It can be observed that physiological parameters had the main impact on distance calculations, whereas morphological parameters did not influence the hierarchical process.

Table 5. Mean value of each parameter determined for both clusters (tomato plants). Bold *p*-values indicate statistically significant differences between cluster 1 and cluster 2, estimated with Student's *t*-test and $p < 0.05$.

Parameter	Cluster 1	Cluster 2	*p*-Value
% weight change	102	102	0.798161
% height change	94	84	0.493144
Relative leaf number	1.15	1.00	0.557965
F_0	452	511	**0.000249**
Fm	2374	2260	**0.002449**
Fv	1922	1749	**0.000067**
Fv/F_0	4.28	3.61	**0.000000**
Fv/Fm	0.81	0.77	**0.000074**
Tfm	188	209	**0.015459**
Area	19,702	16,086	**0.000004**
Fm/F_0	5.25	4.46	**0.000001**
PI ABS	1.00	0.65	**0.000000**
ABS/RC	3.23	3.85	**0.000323**
TR_0/RC	2.60	2.88	**0.000195**
ET_0/RC	1.07	0.99	**0.015152**
DI_0/RC	0.62	0.98	**0.001677**
ET_0/ABS	0.33	0.27	**0.000000**
ET_0/TR_0	0.41	0.35	**0.000000**

Cluster 1 mostly consisted of plants from Control groups of tomato accessions, revealing favorable values of tested parameters, whereas Stress groups were mostly assigned to Cluster 2. However, in Cluster 1, there were control and stress-treated plants from the three accessions (PZ 715, POL 8/15, and POL 7/15); this meant that waterlogging stress did not have negative impact on changes in the parameters. According to this, accessions PZ 715, POL 8/15, and POL 7/15 were considered as more tolerant to oxygen deprivation. Going further in the classification, we conducted a comparison of parameters between Control and Stress groups in the accessions selected above (Table 6). Statistical analysis indicated that in POL 8/15, eight parameters changed under waterlogging stress, whereas in POL 7/15, only two of all estimated parameters were unstable. Therefore, accession POL 7/15 was selected as the most tolerant tomato accession to waterlogging.

When searching for more sensitive accessions, we chose those included in both clusters and with a large Euclidean distance. The Euclidean distance matrix depicts the Control and Stress groups of the PZ 215 accession that were furthest apart and, as a result, were selected as more sensitive (Figure 6). Moreover, we selected PZ 115 Control and Stress groups with large distance. Both selected accessions are compared in Table 7. Controls of both presented accessions were included in cluster 1 and those under Stress treatment in cluster 2. However, more parameters were changed after stress treatment in the case of PZ 215. Figure 6 demonstrates elements of the Euclidean distance matrix of tested accessions and confirms POL 7/15 and PZ 215 as accessions with an opposite response to oxygen deprivation.

Table 6. Mean value of each parameter determined in three tomato accessions considered as more tolerant to waterlogging. Bold *p*-values indicate statistically significant differences between Control and Stress plants of each accession separately estimated with Student's *t*-test and $p < 0.05$.

Parameter	POL 7/15 Control	POL 7/15 Stress	*p*-Value	POL 8/15 Control	POL 8/15 Stress	*p*-Value	PZ 715 Control	PZ 715 Stress	*p*-Value
% weight change	100	117	**0.0005**	100	103	0.6168	100	105	**0.0282**
% height change	100	70	**0.0239**	100	72	**0.0255**	100	81	0.0537
Relative leaf number	1.10	1.05	0.8010	0.95	1.00	0.7699	1.00	0.68	0.0671
F_0	441	447	0.6030	429	453	0.2131	439	496	**0.0002**
Fm	2259	2317	0.5088	2416	2324	0.1287	2292	2468	**0.0413**
Fv	1818	1869	0.5695	1987	1871	0.0507	1853	1972	0.1355
Fv/F_0	4.17	4.20	0.9211	4.65	4.22	**0.0411**	4.22	3.99	0.1858
Fv/Fm	0.80	0.81	0.5391	0.82	0.80	**0.0417**	0.81	0.80	0.1699
Tfm	178	191	0.3619	187	213	0.1696	177	196	0.3126
Area	19,874	20,181	0.8558	20,194	17,879	0.0592	19,530	19,283	0.8493
Fm/F_0	5.17	5.20	0.9215	5.65	5.22	**0.0411**	5.22	4.99	0.1862
PI ABS	1.13	0.95	0.3583	1.32	0.94	**0.0108**	0.90	0.82	0.3596
ABS/RC	3.15	3.18	0.8164	2.97	3.37	**0.0003**	3.49	3.35	0.1196
TR_0/RC	2.51	2.56	0.3718	2.44	2.71	**0.0002**	2.82	2.67	**0.0495**
ET_0/RC	1.04	1.05	0.8469	1.08	1.11	0.4794	1.19	1.08	**0.0013**
DI_0/RC	0.65	0.62	0.6056	0.53	0.66	**0.0035**	0.67	0.68	0.9076
ET_0/ABS	0.34	0.33	0.7765	0.36	0.33	0.0726	0.34	0.32	0.1381
ET_0/TR_0	0.42	0.41	0.7011	0.44	0.41	0.1028	0.42	0.40	0.1901

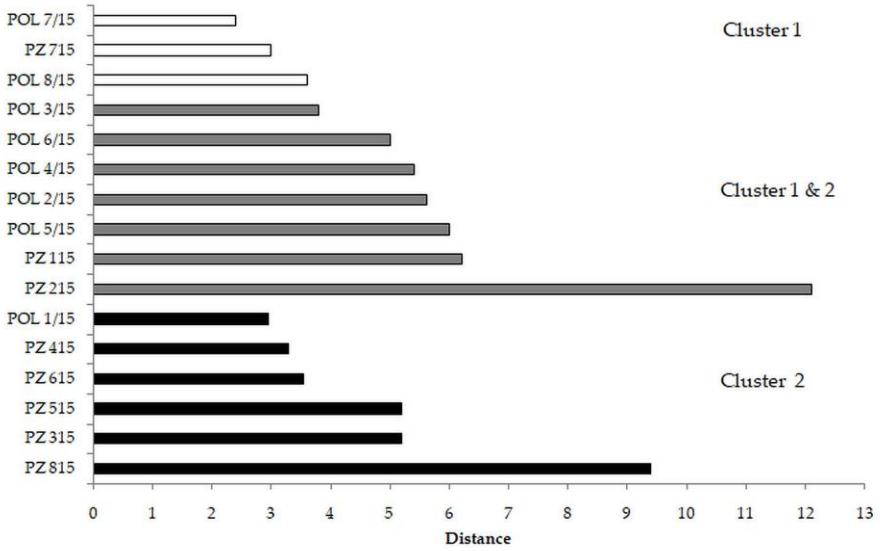

Figure 6. Euclidean distance between Control and Stress groups of each tomato accession. White bars indicate accessions from control and stress treatments that were classified into cluster 1; light grey bars indicate accessions from control and stress treatments that were classified into different clusters; black bars indicate accessions from control and stress treatments that were classified into cluster 2.

Table 7. Mean value of each parameter determined in two tomato accessions considered as more sensitive to waterlogging. Bold *p*-values indicate statistically significant differences between Control and Stress plants of each accession separately estimated with Student's *t*-test and $p < 0.05$.

Parameter	PZ 115 Control	PZ 115 Stress	*p*-Value	PZ 215 Control	PZ 215 Stress	*p*-Value
% weight change	100	109	**0.0160**	100	109	**0.0332**
% height change	100	211	**0.0001**	100	11	**0.0000**
Relative leaf number	0.00	0.20	0.0527	0.53	0.16	**0.0160**
F_0	454	491	0.1741	456	677	**0.0069**
Fm	2502	2274	**0.0065**	2371	2211	0.0645
Fv	2048	1783	**0.0051**	1915	1534	**0.0053**
Fv/F_0	4.54	3.80	**0.0149**	4.22	2.82	**0.0014**
Fv/Fm	0.82	0.78	**0.0328**	0.81	0.68	**0.0034**
Tfm	210	259	0.0775	183	244	**0.0000**
Area	19,309	15,832	0.0864	20,171	13,332	**0.0010**
Fm/F_0	5.54	4.80	**0.0149**	5.22	3.82	**0.0014**
PI ABS	1.10	0.73	**0.0201**	0.97	0.43	**0.0008**
ABS/RC	3.46	3.89	**0.0448**	3.20	5.13	**0.0009**
TR_0/RC	2.82	3.00	0.0866	2.58	3.22	**0.0000**
ET_0/RC	1.21	1.15	0.0975	1.04	0.97	0.3251
DI_0/RC	0.64	0.89	**0.0445**	0.62	1.91	**0.0051**
ET_0/ABS	0.36	0.31	**0.0313**	0.33	0.23	**0.0019**
ET_0/TR_0	0.43	0.39	**0.0466**	0.41	0.31	**0.0036**

As a summary, the radar charts were created for POL 7/15 and PZ 215 tomato accessions, defined as more tolerant and more sensitive, respectively (Figure 7). The radar charts strongly highlighted the differences in response to waterlogging stress in selected tomato accessions. There were statistically significant differences in the weight and height of plants between the Control and Stress groups in POL 7/15, and thus only morphological parameters changed. In case of PZ 215, only 2 of 18 parameters were stable: Fm and ET_0/RC (Table 7 and Figure 7).

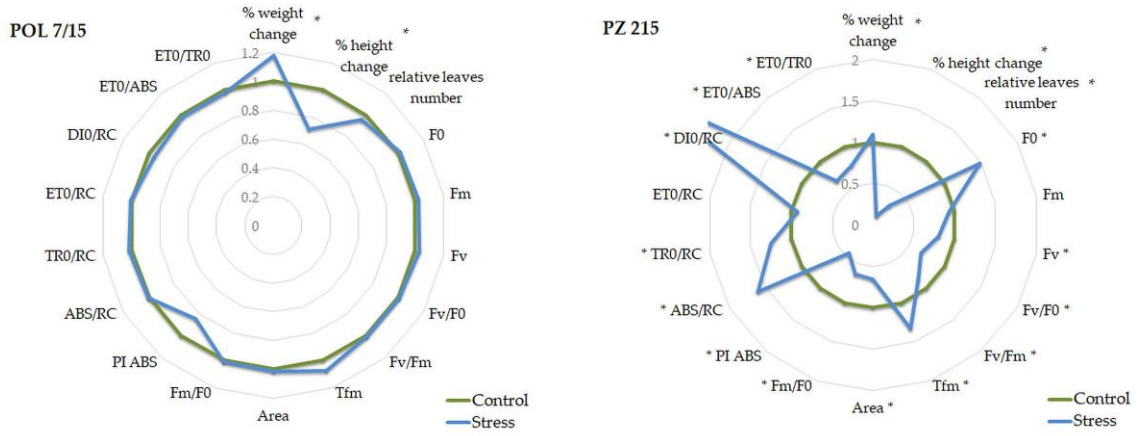

Figure 7. Radar charts comparing 18 traits estimated in Control and Stress plants of two tomato accessions POL 7/15 and PZ 215. Parameters from the Control group were set as 1 and parameters of Stress-treated plants were expressed in relation to Control. Asterisks indicate significant differences between Control and Stress plants according to Student's *t*-test and $p < 0.05$, calculated separately for each parameter.

4. Discussion

The reaction of tomato or cucumber accessions to waterlogging stress is diversified. As we have shown, accessions can be grouped for those whose parameters significantly worsen after stress and those that do not show a significant deterioration in functioning. In the presented research, we focused on the response of the aerial part to stress present within the root system. During hypoxia of the root system, signals to the aboveground part—often found in optimal oxygen conditions—are transmitted within the plant body [47–49]. The signal that moves from the root to the aboveground part during

hypoxia stress changes the functioning of the shoots. The most important process in the aboveground part of plants is photosynthesis, which generates energy and carbohydrates. Stress conditions affect photosynthesis (see the review in [50]). Chloroplasts, key organelles for photosynthesis, are highly sensitive to many stress factors. The photosynthesis process can be disrupted due to decreases in pigment content, changes in electron transport, or disorders in the activities of enzymes related to CO_2 fixation. Moreover, limitations in gas diffusion (CO_2 and water) can be observed due to stomata closure. Therefore, it is reasonable to study the intensity of photosynthesis or chlorophyll *a* fluorescence during stresses involving the root system, such as hypoxia, salinity, or others that interfere in the functioning of the plant. For example, decreases in net photosynthesis as well as a decline in maximal photochemical efficiency of PSII after hypoxia have been observed in sensitive accessions of cotton [39], while in tolerant forms, the changes were not observed. The JIP-test, widely discussed since 1995, is a good tool for comparing stressed and control samples and allows investigation of the effects of almost any stress factor, although only if the stress affects photosynthesis [51]. For example, Kalaji et al. [52] tested the impact of 14 abiotic stress factors on barley by fast chlorophyll fluorescence kinetics and used this method as a crop phenotyping tool. Chlorophyll fluorescence measurement is a fast and non-destructive method to analyze the photosynthetic apparatus [53], allowing for the detection of stress effects before the visible signs are noticed [52]. Thus, in our research, we chose parameters related to plant growth and chlorophyll *a* fluorescence in the leaves.

After performing statistical analysis, we divided the studied waterlogged and control accessions into clusters. In both species, significant decreases in Fm, Fv, Fv/F_0, Fv/Fm, Area, Fm/F_0, PI ABS, ET_0/RC, ET_0/ABS, and ET_0/TR_0 parameters were observed in cluster 2 as compared to cluster 1. In addition, increases in DI_0/RC was observed. According to this information, we conclude that in cluster 1, plants had parameters with more favorable values, and in cluster 2, plants had worse parameters (Table 2, Table 5, and Table S1). The decrease in Fm or Fv/Fm is connected with a lower ability of PSII to reduce the QA primary acceptor [54]. Furthermore, the decrease in Fv/F_0 during stress could be an indicator of lower efficiency of photochemical processes in PSII [55]. The Area parameter represents the pool size of electron acceptors in PSII, with this pool size being lower during reductions in electron transport in submergence stress [54]. The lower value of ET_0/RC is also indicator of disturbances in electron transport from QA to other acceptors. Panda et al. [54] state that both the donor and the acceptor side of PSII were damaged because of submergence. The PI ABS index includes information about the probability that the chlorophyll *a* molecule functions as a reactive center in PSII, the efficiency of transfer of the absorbed energy to the reduction of QA, and the probability that an electron moves further than QA. It is a very sensitive parameter that decreases during stress conditions [51]. When the photochemistry of photosynthesis is disrupted by stress factors, the dissipation of absorbed energy increases [56], and this can be observed as increase in the DI_0/RC value.

Considering the results of the cluster analysis, we chose a more tolerant and more sensitive accession in both species. The parameters of more tolerant accessions did not deteriorate after appropriate stress, and the Control and Stress group of such plants were in cluster 1. In the case of more sensitive accessions, the parameters significantly deteriorated, and the Control group was in cluster 1 and the Stress-treated group in cluster 2. The changes in OJIP test parameters after submergence stress were more pronounced in sensitive rice (*Oryza sativa* L.) cultivars than in tolerant ones [54]. Similarly, in our experiments, more significant differences, indicating the deterioration of the photosynthetic apparatus, between Stress and Control plants could be observed in the case of sensitive accessions compared with that in more tolerant accessions (Figures 4 and 7). For example, the Fv/F_0, Fv/Fm, Area, Fm/F_0, PI ABS, and DI_0/RC parameters remained at the same level after stress treatment in the case of more tolerant accessions, but changed in sensitive accessions after stress. Increases in DI_0/RC in more sensitive accessions of both species indicated that some of the absorbed energy was dissipated and not used in the photochemistry of photosynthesis. In the case of more tolerant plants, DI_0/RC was stable. In agreement with our observation of more sensitive samples, we observed increases in DI_0/RC parameter in cucumber plants after hypoxia stress [56].

The decrease in Fv/Fm was noted in more sensitive tomato and cucumber plants in our experiments. In agreement with our results, a decrease in Fv/Fm after waterlogging was also observed in rice [54,57], cucumber [56,58], tomato [59,60], wild tomato (*Solanum habrochaites* S.Knapp & D.M.Spooner) [61], Arabidopsis (*Arabidopsis thaliana* L.) [62], cotton [39], and pepper (*Capsicum annuum* L.) plants [63]. Barik et al. [57] examined the reaction of tolerant and susceptible varieties of rice to submergence and observed that the latter exhibited a greater reduction in Fv/Fm parameters in comparison to tolerant varieties. Similarly, in an experiment with cotton, the Fv/Fm parameter was stable in tolerant varieties and significantly decreased in sensitive varieties under hypoxia [39]. In the case of sorghum after waterlogging stress, the changes in Fv/Fm parameters were not significant, despite the observation of a substantial decrease in the Fv/F_0 parameter [64]. In the present experiment, the decrease in Fv/Fm in more sensitive tomato genotypes was about 16%, and for the Fv/F_0 parameter, this was about 33% after stress treatment; in sensitive cucumber genotypes, these figures were 2.4% and 11.5%, respectively. This suggests that Fv/F_0 is a more sensitive parameter than Fv/Fm, consistent with the findings of Tsimilli-Michael [51]. However, the parameter Fv/Fm is more often described in the literature. Kalaji et al. [52] observed that PI ABS is the most sensitive parameter to different stress conditions. A decrease in this parameter was observed under waterlogging stress in terms of rice [54], cucumber [56], or tomato [59]. In the present experiments, the decrease in PI ABS in the case of sensitive tomato plants was about 56%, and for cucumber plants this was about 28% after stress treatment. This parameter did not change in more tolerant accessions after waterlogging stress. Our observations confirm the high sensitivity and usefulness of this parameter in plant selection to waterlogging stress.

Many studies have presented results of plant morphological observations after waterlogging stress; however, sometimes inconsistent information can be found. Decreases in plant height were observed after waterlogging in cucumber [56] and field bean (*Vicia faba* L. minor) [65]. Six cotton varieties, sensitive and tolerant to hypoxia stress, were tested by Pan et al. [39], and an inhibition in plant growth was observed in more sensitive varieties. However, plant height in stressed tolerant cotton was similar to untreated plants. In the terms of fresh mass of plants, we observed a decrease in terms of cucumber [58], barley [38], and pepper [63] after stress treatment, but no changes were observed by He et al. [56] in cucumber fresh weight after hypoxia. In our results, some accessions indicated an increase in morphological parameters, whereas others decreased. It is worth mentioning that both selected accessions of cucumber and tomato (tolerant and sensitive) demonstrated a decrease in plant height after stress treatment. The appearance of selected accessions is presented in Figure S3. The inhibition of growth does not seem to be correlated with the activity of PSII.

Using physiological parameters and cluster analysis, Barik and co-workers [57] classified seven rice cultivars into two clusters. Cluster one included submergence-tolerant rice varieties whereas susceptible varieties were included in cluster two. These data indicated the usefulness of cluster analysis in stress tolerance classification. Moreover, in our experiment, cluster analysis helped in classification, although the procedure was slightly different. Cluster analysis can be used in stress tolerance plant classification, as also demonstrated by Cao et al. [40], wherein the authors used cluster analysis to divide tomato genotypes into those that are more or less tolerant to chilling stress.

As part of our cooperation with Polish breeders, we also conducted a sensitivity assessment of homozygous cucumber lines for waterlogging stress and selected more tolerant and more sensitive lines (see the Supplementary Materials Section). Selected cucumber homozygous lines can be used for basic research on stress resistance or for breeding new varieties adapted to new breeding programs. The results can be of use not only to Polish breeders, but also to international breeders. According to previous information, choosing more tolerant accessions maintained better PSII activity.

Two tools were used in the present work: chlorophyll *a* fluorescence was applied as the main tool to assess the state of the photosynthetic apparatus of stress-treated plants and statistical analysis was used to select sensitive and tolerant accessions on the basis of the obtained empirical data. The selected objects will be used for further analysis related to understanding the mechanisms of the stress response to hypoxia in tomato and cucumber plants. In future research, we plan to evaluate the effect of

waterlogging of selected tomato and cucumber plants on the photosynthetic rate, chlorophyll and carotenoid accumulation, and other parameters related to the functioning of leaves. We will also investigate if there are differences between selected accessions in terms of yield quality and quantity during stress.

5. Conclusions

Chlorophyll *a* fluorescence can be used for the selection of plant accessions sensitive to waterlogging stress.

Not all parameters of the OJIP test seem to have the same sensitivity; Fv/F_0, PI ABS, as well as DI_0/RC or Area appear to be better in selecting sensitivity to waterlogging stress than Fv/Fm.

From the tested accessions, we selected GM-50, POL 7/15, and DH2 as more tolerant, whereas TYTUS, PZ 215, and DH4 were determined to be more sensitive for waterlogging stress.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/10/10/1490/s1, Figure S1: Results of cluster analysis for cucumber homozygous lines using the Euclidean distance on the basis of morphological and physiological traits (Ward's hierarchical algorithm); green line indicates the cut-off point. Table S1: The mean values of parameters determined for each cluster and statistical comparison between clusters estimated in cucumber homozygous lines (bold p-values mean statistically significant differences between cluster 1 and cluster 2). Figure S2: Euclidean distance between Control and Stress groups of each cucumber homozygous lines. White bars indicate accessions from control and stress treatments that were classified into cluster 1; light grey bars indicate accessions from Control and Stress treatments that were classified into different clusters; black bars indicate accessions from Control and Stress treatments that were classified into cluster 2. Figure S3: Accessions GM-50, POL 7/15, and DH2 selected as more tolerant, and TYTUS, PZ 215, and DH4 determined to be more sensitive for waterlogging stress. C: Control plants, S: Stress-treated plants.

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Long-Term Waterlogging as Factor Contributing to Hypoxia Stress Tolerance Enhancement in Cucumber: Comparative Transcriptome Analysis of Waterlogging Sensitive and Tolerant Accessions

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Abstract: Waterlogging (WL), excess water in the soil, is a phenomenon often occurring during plant cultivation causing low oxygen levels (hypoxia) in the soil. The aim of this study was to identify candidate genes involved in long-term waterlogging tolerance in cucumber using RNA sequencing. Here, we also determined how waterlogging pre-treatment (priming) influenced long-term memory in WL tolerant (WL-T) and WL sensitive (WL-S) i.e., DH2 and DH4 accessions, respectively. This work uncovered various differentially expressed genes (DEGs) activated in the long-term recovery in both accessions. De novo assembly generated 36,712 transcripts with an average length of 2236 bp. The results revealed that long-term waterlogging had divergent impacts on gene expression in WL-T DH2 and WL-S DH4 cucumber accessions: after 7 days of waterlogging, more DEGs in comparison to control conditions were identified in WL-S DH4 (8927) than in WL-T DH2 (5957). Additionally, 11,619 and 5007 DEGs were identified after a second waterlogging treatment in the WL-S and WL-T accessions, respectively. We identified genes associated with WL in cucumber that were especially related to enhanced glycolysis, adventitious roots development, and amino acid metabolism. qRT-PCR assay for hypoxia marker genes i.e., *alcohol dehydrogenase* (*adh*), *1-aminocyclopropane-1-carboxylate oxidase* (*aco*) and *long chain acyl-CoA synthetase 6* (*lacs6*) confirmed differences in response to waterlogging stress between sensitive and tolerant cucumbers and effectiveness of priming to enhance stress tolerance.

Keywords: *Cucumis sativus* L.; DEGs; gene expression; hypoxia; priming; RNA-Seq; transcriptome; waterlogging

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1. Introduction

Among the negative effects of climate change are more frequent violent downpours that cause local flooding, which, in turn, lead to long-term waterlogging of plants. As a consequence, this means a reduction in oxygen availability in the plant root zone, i.e., hypoxia [1–3]. Additional factors that trigger long-term hypoxia in the root system are compacted soil and poor substrate aeration [4], even in drought areas [5]. Hypoxic stress in plants may also occur in hydroponic greenhouse cultivation, where oxygen availability decreases due to inadequate irrigation of the root system and insufficient aeration of the flowing medium [6].

Cucumber (*Cucumis sativus* L.), due to its shallow root system [7], is considered as being sensitive to the stress of limited oxygen access [6,8–10]. Differences in the response to oxygen deprivation between waterlogging (WL) tolerant (WL-T) and WL sensitive (WL-S) accessions using the RNA-Seq approach were evaluated in *Arabidopsis thaliana* [11], rice (*Oryza sativa*) [12], rapeseed (*Brassica napus* L.) [13], soybean (*Glycine max*) [14], kiwifruit (*Actinidia deliciosa*) [15], sesame (*Sesamum indicum* L.) [16], and cucumber [17]. However,

these studies focused mainly on the response to hypoxic stress in the initial stages; there are no data from RNA-Seq studies carried out in cucumber roots regarding long-term waterlogging in WL-S and WL-T accessions. Understanding the mechanisms involved in the response to hypoxia in tolerant genotypes will enable the development of cultivars resistant to excess water in the soil in areas at risk of waterlogging [10].

When the stress is over, the plant shifts into the recovery period [18]. Yeung et al. [19] proposed a signaling network that participates in the recovery period after waterlogging in plants. According to these authors, ROS (Reactive Oxygen Species), ABA (Abscisic acid) and ethylene are the first signals evoking various pathways during the recovery period. In turn, increased activity of SOD (Superoxide dismutase) and CAT (Catalase) was demonstrated in cucumber leaves in WL-T cucumber, whereas in WL-S activity of those enzymes was decreased after 7 days of waterlogging [20]. Nonetheless, the molecular mechanisms involved in the long-term recovery period after waterlogging in cucumber roots are still unknown. We wanted to better understand the pathways engaged in a long-term root recovery period in WL-S and WL-T cucumber accessions, which is very important in the context of future research on plant waterlogging tolerance [18,20].

It is known that plants have a stress memory that facilitates them overcoming recurring exposure to stress [21]. Primed plants, as a consequence of acquired stress memory [22], are more tolerant and able to respond faster and more effectively when they are exposed to stress factors again [23–25].

In cucumber, there are only a few papers describing the phenomenon of priming. Usage of melatonin as a priming factor has been studied against salt and water stress [26,27] and *Rhizobacterium* strains have been used against drought stress [28]. Still, the usage of waterlogging as a priming factor against oxygen deprivation in cucumber is not known. Priming and stress memory, as a consequence, are one of the main approaches developed by breeders for use in resistant plant production [29–31], either as an alternative to genetically modified (GM) plants or in beneficial financial terms [32]. Still, many questions remain unanswered, even those concerning the duration of memory in the life of a plant or the application of priming in the field conditions, not only in the laboratory.

In this work, we performed transcriptomic analysis using RNA-Seq to identify metabolic pathways regulated under long-term waterlogging treatment in both WL-T DH2 and WL-S DH4 cucumber accessions. Secondly, we wanted to determine how priming (pre-treatment) influences long-term memory in WL-T and WL-S cucumbers. Additionally, this work aimed to assess the effect of waterlogging on the long-term recovery period in cucumber roots.

2. Materials and Methods

2.1. Plant Material and Experiment Conditions

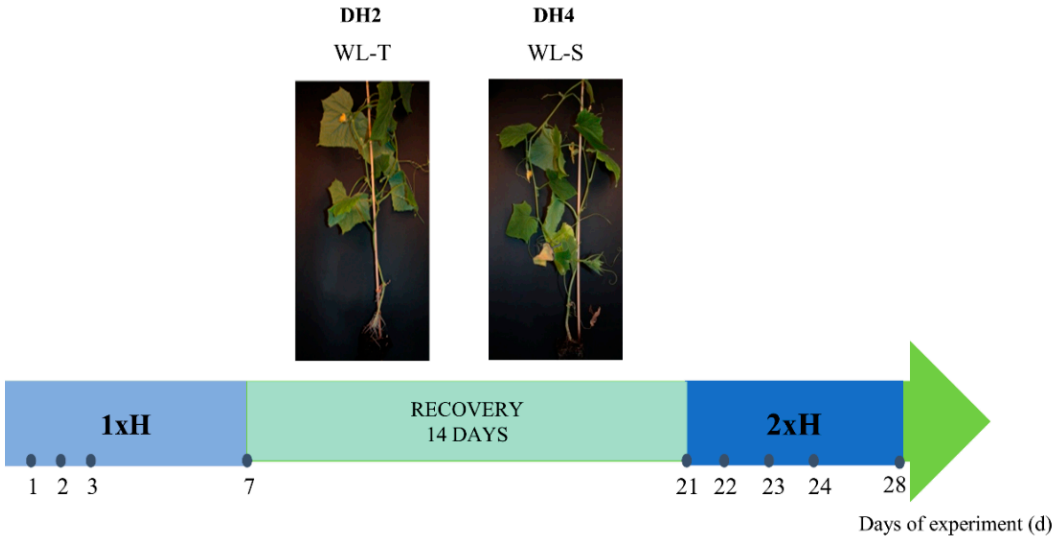
Seeds of two double-haploid (DH) lines of cucumber, i.e., DH2 (WL-T) and DH4 (WL-S), provided by KHiNO Polan (Poland), with contrasting responses to oxygen deprivation in the soil [10], were sown in 40-cell multi-pots (0.23 dm^3 each cell) which were filled with Klasmann KTS-2 peat substrate (Klasmann; Geeste, Germany) containing (in mg dm^{-3}) 250–500 N, 170–230 P_2O_5, 320–500 K_2O, and 80–120 Mg. The plants were cultivated under controlled conditions in a greenhouse and were lit with supplementary radiation (high pressure sodium lamps) to sustain a 16/8 h light/dark regime at 27 °C during the day and 24 °C during the night. Minimum photosynthetic photon flux density (PPFD) on plant level during the day was 80 ± 20 µmol m^{-2} s^{-1}. All plants were fertilized with growth fertilizer 3 days before the first waterlogging and every 3 days from the end of the first waterlogging stress with regenerative fertilizer. The growth fertilizer contained 7.5 g of Superba^{TM} Green Forte (8.2% N, 11.5% P_2O_5, 36.1% K_2O, 2.8% MgO, 5.7% S, 0.23% Fe, 0.14% Mn, 0.03% Zn, 01% Cu, 0.04 B, 0.003% Mo) (Yara International ASA; Oslo, Norway), 7.0 g of YaraLiva CALCINIT Flakes (15.2% N, 27.5% CaO) (Yara International ASA; Oslo, Norway), and 3.0 g KRISTA^{TM} MAG (11% N, 15% MgO) (Yara International ASA; Oslo, Norway) diluted in 10 dm^3 H_2O, whereas the regenerative fertilizer consisted of 11.5 g,

8.8 g, and 4.4 g, respectively, of the same components as the growth fertilizer. The pH of the fertilizers was adjusted to 5.8 with nitric acid (V), and the final soil electrical conductivity (EC) was 2.8–3.1 ms cm^{-1}; 25 mL of fertilizer was applied to each pot.

2.2. Stress Treatment

After 21 days of cultivation, plants were divided into three groups: untreated plants, cultivated under optimal conditions (Ctrl), non-primed plants, waterlogged for 7 days only once, (1xH), plants after 7 days of waterlogging and 14 days of recovery (Rec), and primed plants waterlogged for 7 days and after 14 days of recovery, then waterlogged again (2xH) (Figure 1).

The percent volumetric water content (VWC) was measured using a Delta-T Devices SM150 soil moisture sensor kit (Delta-T Devices Ltd.; Cambridge, UK) before stress treatment and, if needed, plants were watered to obtain a soil moisture level up to 30%. The root zone and hypocotyls at a height of around 4–7 cm of the plants in the 1xH group and 2xH group were waterlogged for 7 days in deep plastic trays (600 × 400 × 200 cm^3). Then, plants were taken out of the water and stayed unstressed for 14 days (Rec). Later on, half of the stressed plants from the 1xH group were waterlogged for the second time for another 7 days (2xH) (Figure 1). The plants from the control group (Ctrl) stayed unstressed throughout the experiment and were watered as needed to ensure optimal growth conditions. Oxygen levels in the water (for waterlogged plants) and in the air (for control plants) were periodically monitored using a dissolved oxygen (DO) meter (HI 2040-02 edge, Hanna instruments; Woonsocket, RI, USA). During waterlogging, the oxygen level in the water reached 2.5 mg dm^{-3}, which confirmed hypoxic condition [33], whereas in the air, the dissolved O_2 level was 9.0 mg dm^{-3}.



	1	2	3	7	21	22	23	24	28
Morphological measurments				✓	✓				✓
RNA-Seq				✓	✓				✓
qRT-PCR	✓	✓	✓	✓	✓	✓	✓	✓	✓

Figure 1. Scheme describing the experiment duration and time-points of sample collection for morphological parameter measurements, RNA-Seq and qRT-PCR. The image presents double haploid (DH) lines of 1xH treatment i.e., WL-T DH2 and WL-S DH4.

2.3. Measurement of Morphological Parameters

Effect of waterlogging stress on vegetative growth was estimated by comparing growth parameters between control and stressed plants. Before the waterlogging treatment, 20 random plants from three experimental groups i.e., Ctrl, 1xH, and 2xH, were labeled for plant height and leaf number. Plant height [cm], number of leaves, and root and shoot fresh weight (FW, g) and dry weight (DW, g) were measured at 7, 21, and 28 d of the experiment. Plant heights were measured from the base of the plant (above-ground) to the meristem. The weight of adventitious roots [g] (if developed) was also estimated. For the dry weight (DW) determination, the plant material was dried at 105 °C in an oven for 24 h and weighed afterwards.

2.4. RNA Extraction for RNA-Seq and qRT-PCR

For RNA-Seq analysis, the roots of both cucumber DH lines were collected from Ctrl, 1xH, Rec, and 2xH plants (Figure 1). Material from the control group was collected at 7, 21, and 28 d of the experiment and was pooled into 3 biological replicates, whereas single replicate contained the roots from 5 independent plants from each time point. For stressed plant groups (1xH, Rec, and 2xH), 3 biological replicates were prepared, whereas each of them pooled roots from 5 plants.

For the qRT-PCR assay, the roots were collected at 1, 2, 3, and 7 d from the control, 1xH, Rec, and 2xH groups at each time-point of the experiment.

Roots were collected, washed carefully in clean water, frozen immediately in liquid nitrogen, and then stored at −80 °C until RNA extraction. Total RNA isolation was performed with Direct-zol RNA MiniPrep Plus (Zymo Research, Irvine, CA, USA) according to the manufacturer's instruction. All RNA extracts were treated with 1 U μL^{-1} RNase-free *Dnase* I (Thermo Fisher Scientific, Waltham, MA, USA) and 40 U μL^{-1} of RiboLock RNase Inhibitor (Thermo Fisher Scientific, Waltham, USA) to prevent DNA contamination. RNA quality and quantity were monitored by gel electrophoresis under denaturing conditions. The A260/A280 ratio and RNA integrity number (RIN) were determined by a Bioanalyzer 2100 (Agilent 2100 Bioanalyzer; Agilent Technologies, Palo Alto, Santa Clara, CA, USA).

2.5. RNA Library Construction and Illumina Sequencing

The cDNA libraries were prepared using the NEBNext® Ultra™ RNA Library Kit (Illumina, San Diego, CA, USA). In total, 24 cDNA libraries, i.e., two cucumber accessions (WL-T DH2 and WL-S DH4), x4 treatments (Ctrl, 1xH, Rec, 2xH), x3 experimental triplicates, were subjected to sequencing in PE101 (paired ends mode, with 101 bp read length) on an Illumina HiSeq4000 (Illumina, San Diego, CA, USA).

The RNA-Seq datasets generated for this study are deposited in the NCBI under BioProject PRJNA678740 and can be found in the GenBank Short Read Archive (SRA) under Acc. No. SSR13083584 to SSR13083607.

2.6. Data Filtering and Quality Control

The raw sequences in FASTQ format were subjected to TRueSeq3-PE adaptor removal using Cutadapt ver. 1.9.1 (http://cutadapt.readthedocs.io). Quality trimming was processed using BBDuk2 from the BBMap toolkit ver. 37.02 (https://jgi.doe.gov/data-and-tools/bbtools). FASTQC ver. 0.11.5, (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was applied for standard pre- and post-trimming quality control. To pass the quality filter, read quality needed to surpass a Phred score (Q) of 20 and achieve a minimal length of 50 bp; all unpaired reads were excluded. *C. sativus* rRNA sequences were downloaded from the 5s rRNA database (http://combio.pl/rrna) and Bowtie2 ver. 2.3.3.1 (http://bowtie-bio.sourceforge.net/bowtie2/index.shtml) was employed for mapping and discarding 5s rRNA sequences from the set of data.

2.7. Mapping to the Reference Genome

The high-quality reads were aligned using STAR ver. 2.5.3a [34] (parameters: outSAMmapqUnique: 50; outSAMattributes: all; outSAMtype BAM SortedByCoordinate; outSAMstrandField: intronMotif; outFilterIntronMotifs: RemoveNoncanonical; outFilterType: BySJout; outFilterMultimapNmax: 20; alignSJoverhangMin: 8; alignSJDBoverhangMin: 1; alignIntronMin: 20; alignIntronMax: 1,000,000; alignMatesGapMax: 1,000,000; chimSegmentMin: 12; chimJunctionOverhangMin: 12; chimSegmentReadGapMax: 3) to the reference genome (ftp://cucurbitgenomics.org/pub/cucurbit/genome/cucumber/Chinese_long/v2) downloaded from the Cucurbit Genomics Database at http://cucurbitgenomics.org published by Huang et al. [35]. The alignment data were used to calculate the distribution of reads for the reference genes and to perform coverage analysis.

2.8. Transcriptome de novo Assembly

De novo transcriptome assembly for each biological replicate was performed using StringTie ver.1.3.3b [36] with the following parameters: minimum assembled transcript length: 200, minimum reads per bp coverage: 10, minimum junction coverage: 10. Cuffmerge and Cuffdiff programs, parts of the Cufflinks package ver.2.2.1 (http://cole-trapnell-lab.github.io/cufflinks) were used to merged the reference annotation and to estimate the transcript abundance. TransDecoder program ver. 5.0.1 [37] was used to identify the coding regions within the transcripts.

2.9. Differential Expression Estimation

Gene expression levels for each sample were estimated by RSEM ver. 1.3.0 (https://deweylab.github.io/RSEM) [38]. Differentially expressed genes were identified using two R packages, i.e., edgeR ver. 3.20.1 [39] and DeSeq2 ver. 1.18 [40] using the p-value correction by Benjamini and Hochberg for the number of repetitions. For further analysis, transcripts identified both as differentially expressed in edgeR and DeSeq2, considering FDR (false discovery rate) < 0.05, were selected (Supplementary Materials Figure S1). Differential gene expression analysis was performed according to the scheme presented in Figure 2. One of the main goals was to compare responses to waterlogging stress between WL-S DH4 and WL-T DH2 treated once (1xH), for that purpose, we compared DH2 1xH vs. DH2 Ctrl and DH4 1xH vs. DH4 Ctrl. Secondly, we wanted to identify DEGs regulated by the second waterlogging treatment (2xH), so that we performed following comparisons: DH2 2xH vs. DH2 Ctrl, DH4 2xH vs. DH4 Ctrl, DH2 1xH vs. DH2 2xH, and DH4 1xH vs. DH4 2xH. Additionally, we wanted to determine changes at the transcriptomic level in plants after 14 days of recovery, so we compared DH2 Rec vs. DH2 Ctrl and DH4 Rec vs. DH4 Ctrl.

2.10. Functional Analysis

The putative function of assembled transcripts was determined using the Trinotate annotation pipeline ver. 3.1.0 (https://trinotate.github.io), dedicated to de novo assembled transcriptomes [41]. The protein-coding regions in assembled unigenes were predicted using TransDecoder ver. 5.0.1 [37]. The pipeline included searching for nucleotide (BLASTx) and protein (BLASTp) homology, performed against the UniProtKB/Swiss-Prot database [42]. We performed the identification of functional protein domains (HMMER/PFAM) [43,44], the prediction of potential protein signals and transmembrane domains (SignalP/tmHMM) [45]) and a comparison with gene annotation databases, i.e., the eggNOG [46], Gene Ontology (GO) [47], and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases (http://www.genome.jp/kegg/) [48]. All annotations were loaded into the Trinotate SQLite database to generate the final annotation report. The topGO R/Bioconductor package ver. 2.38.1 [49] was implemented to test enrichment annotation terms. The significance of occurrence for a certain GO term was determined using Fisher's exact test with a classic method. Analysis focused on groups of genes enriched for the biological process (BP), molecular function (MF), and cellular compartment (CC)

gene ontologies, considering gene groups as significantly regulated with p-values ≤ 0.05. Genes associated with the response to oxygen deprivation in DH2 and DH4 cucumbers were investigated for gene ontological enrichment of biological processes with a p-value < 0.05 as the cut-off criterion using the R package clusterProfiler ver. 3.6.0 [50] (http://www.bioconductor.org/packages/release/bioc/html/clusterProfiler.html).

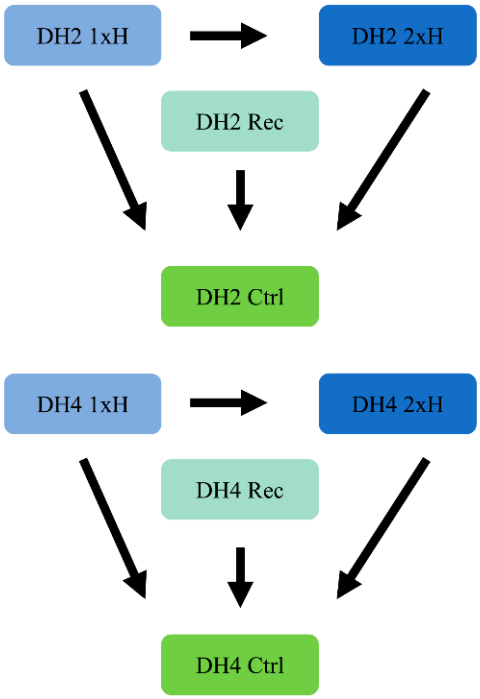


Figure 2. Strategy for differentially expressed genes (DEGs) identification. The arrow direction depicts the comparisons carried out in the analysis.

2.11. qRT-PCR Assay

For the qRT-PCR assay, we selected genes regulated by a limited supply of oxygen and in addition associated with hypoxia tolerance in plants [51–53]. Among selected genes were *alcohol dehydrogenase* (*adh*), a gene involved in fermentation, *1-aminocyclopropane-1-carboxylate oxidase* (*aco*), associated with the synthesis of ethylene and involved in adventitious root development, and *long chain acyl-CoA synthetase 6* (*lacs6*), involved in fatty acid metabolism. The purpose of qRT-PCR was to monitor the expression level of these genes throughout the treatment to firstly confirm the hypoxic conditions, and second to evaluate differences between cucumber accessions and depict the highest expression level over time. qRT-PCR was performed on 1, 2, 3, and 7 d plants treated once (1xH) and twice (2xH), and in plants in the Rec group, at 21, 22, 23, 24, and 28 d of the experiment. cDNA was obtained from 1 µg of total RNA using the iScript™ cDNA Synthesis Kit (Bio-Rad laboratories, Hemel Hempstead, UK) following the manufacturers' instructions. cDNA was diluted 1:5 with DNase/RNase-free H_2O and stored at −20 °C. Quantitative real-time PCR was performed with a QuantStudio 3 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). A single technical repeat contained (25 µL) 12.5 µL of Maxima™ SYBR Green/ROX qPCR Master Mix (2X) (Thermo Scientific, Waltham, CA, USA), 5 µM of gene-specific forward and reverse primers, 1.5 µL of cDNA and RNase-free H_2O. All qPCR reactions were run as three biological replicates, each with three technical replicates. No-template controls (NTCs) were included in every qPCR run. The qPCR method described above conforms to the MIQE (The Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines [54]. The qPCR reactions were amplified at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min., with a final dissociation curve analysis to check for the specificity of

amplification. Relative quantification of gene expression was calculated according to the qBase method [55]. *Actin* (*act*) and *tubulin α chain* (*tua*) were used as the endogenous reference genes. The highly specific primers for *aco* and *lacs6* were designed using the IDT-PrimerQuest tool (http://eu.idtdna.com/primerquest/home/index) and validated using IDT-OligoAnalyzer 3.1 (https://eu.idtdna.com/calc/analyzer) considering the absence of primer-dimers and hairpin structures. All the primers used in this study are listed in Supplementary Materials Table S1.

2.12. Statistical Analysis

Student's *t*-test with $p < 0.05$ was used to compare changes in morphological parameters. Comparisons between treatment and control conditions were conducted at 7, 21, and 28 d of the experiment. Two-way analysis of variance (ANOVA) with two factors (time and treatment) followed by Tukey's test ($p \leq 0.05$) were conducted to determine the influence of hypoxia on gene expression levels. Data are presented as the mean value ± SD for each treatment and time point. The analysis was performed using Statictica ver. 12 (Statsoft).

3. Results

3.1. Plant Morphological Traits

The DH2 and DH4 cucumber accessions were assessed as tolerant (WL-T) and sensitive (WL-S), respectively, after 7 days of hypoxia treatment [10]. Here, the aim of analysis of morphological traits was confirmation of differential stress response of both DH cucumber lines. The effects of waterlogging on plant morphology were monitored at 7, 21, and 28 d of the experiment. After 7 days of stress, a reduction in plant height and the number of leaves was found only in the DH4 accession (Figure 3A,B). In plants of the Recovery group (Rec), a decrease in plant height was observed compared to the non-waterlogged plants (Ctrl) in both cucumber lines (Figure 3A,B). A second treatment with waterlogging stress (2xH) resulted in slower plant growth in both cucumber accessions. At 28 days (after 3 weeks of recovery in 1xH plants), no difference in height was observed in WL-T (DH2). A negative effect of single (1xH) and double (2xH) waterlogging treatments on the number of leaves was observed in WL-S DH4, while a decrease in the number of leaves was recorded in WL-T DH2 only after treating the plants for second time with the stress (2xH) (Figure 3C,D). The number of leaves was lower in the Recovery group of WL-S DH4 compared to the non-stressed plants (Ctrl).

Results shown in Figure 3E–H demonstrate that there was no effect of a single waterlogging (1xH) treatment on the DM:FM ratio in leaves and shoots in the two cucumber accessions. There was a significant decrease in the DM:FM ratio in recovered plants (at 21 days of the experiment) in the DH2 and DH4 accessions. However, a second 7-day waterlogging treatment (2xH) in the two DH lines reduced the DM:FM ratio.

In both DH lines, 7 days of waterlogging caused the development of adventitious roots. However, a greater mass of adventitious roots was found in WL-T DH2 compared to WL-S DH4 (Figure 4).

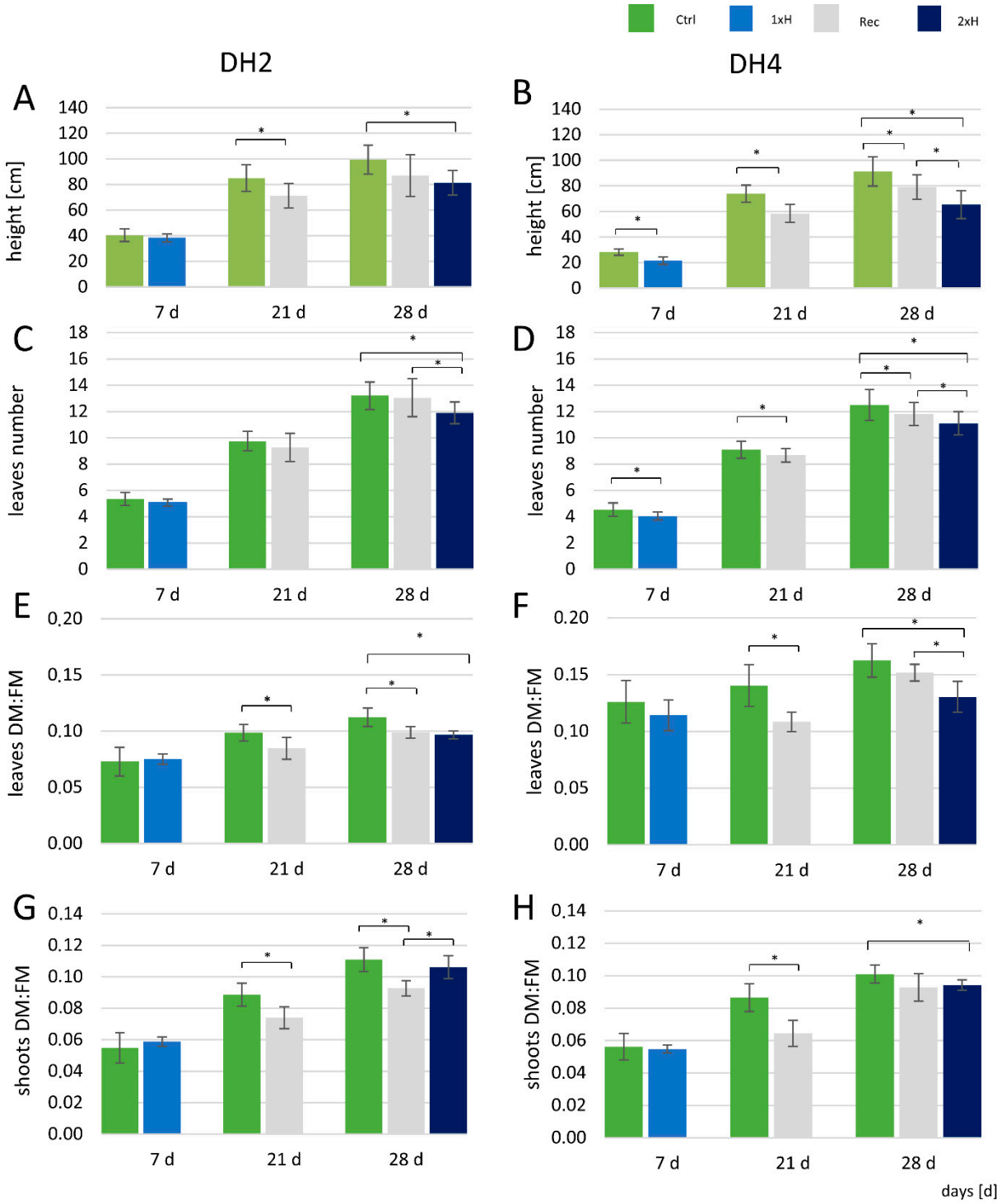


Figure 3. Effect of waterlogging on the plant height (**A**,**B**), leaves number (**C**,**D**), DM:FM ratio in leaves (**E**,**F**) and shoots (without leaves) (**G**,**H**) in DH2 and DH4 cucumber accessions, respectively. DM—dry mass, FM—fresh mass. Values are mean ± standard deviation (n = 10 for plant height and leaf number, n = 6 for FM and DM). * Indicates a significant difference between the indicated treatments at $p \leq 0.05$ according to Student's t-test.

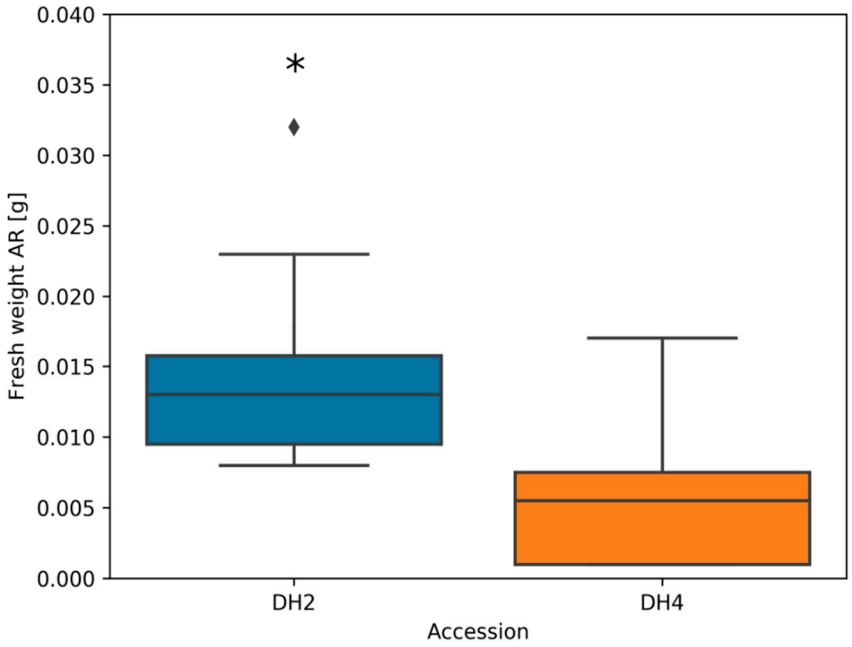


Figure 4. Mass of adventitious roots determined after 7 days of waterlogging in DH2 and DH4 cucumber accessions. Asterix indicates significant differences between accessions (n = 10) according to Student's t-test ($p < 0.05$). The diamond represents the outlier.

3.2. RNA Sequencing and Transcriptome Assembly

The pool of RNA-Seq reads included in total 1,156,427,650 reads (115.2 Gbp), of which 584,234,614 reads (50.5%) were represented by WL-T DH2 and 572,193,036 (49.5%) by WL-S DH4 (Supplementary Materials Table S2). In total, 1,026,182,014 (88.7%) were considered as clean reads.

3.3. Transcriptome Characterization and Functional Annotation

The clean reads represented all samples and were subjected to de novo assembly, resulting in 35,712 transcripts corresponding to 18,093 unigenes (Table 1). The length of the transcripts varied from 162 to 20,773 bp with an average length of 2236 bp. The transcript size distribution showed a high proportion of transcripts in the size range of 800–2200 bp (Figure 5A).

Table 1. Summary of de novo transcriptome data assembly of *Cucumis sativus* L.

	Trascripts
Total numer (bp)	35,712
Total length (bp)	79,877,390
N50 length (bp)	2711
Minimum length (bp)	162
Maximum length (bp)	20,773
Average length (bp)	2236

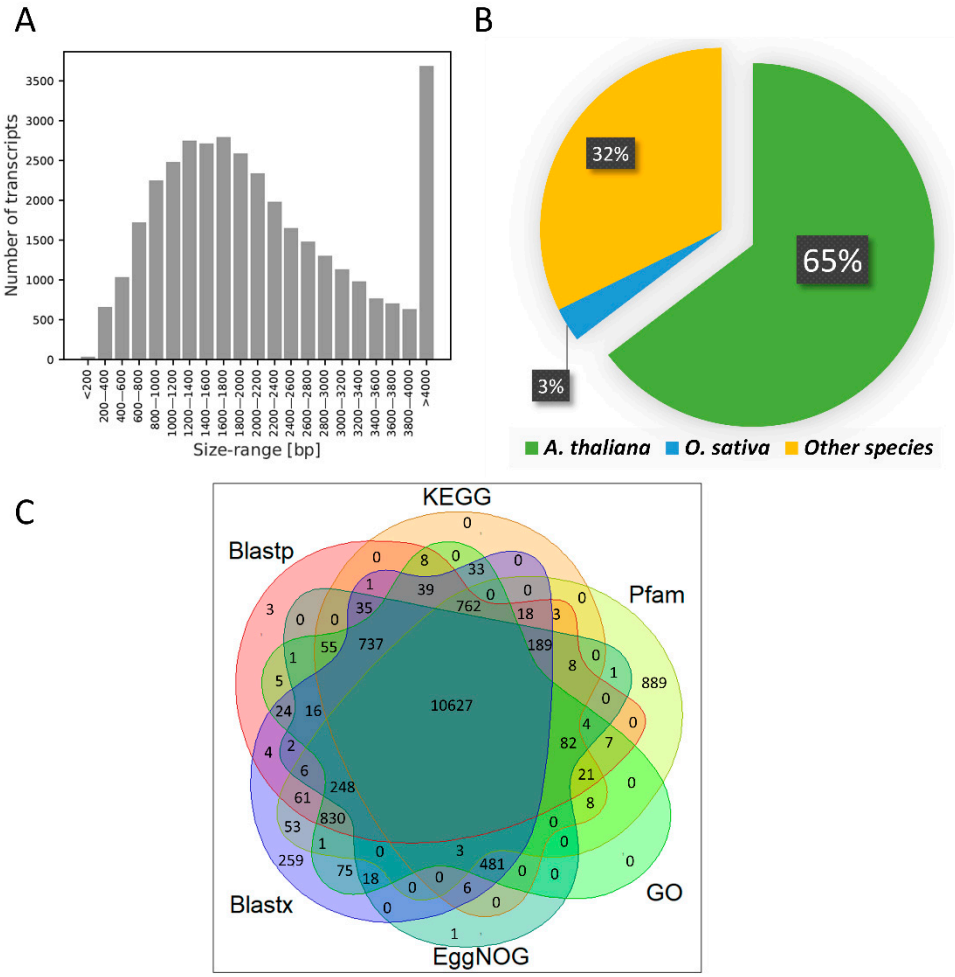


Figure 5. (**A**) Transcript size distribution. (**B**) Homologous species distribution. (**C**) Venn diagram displaying differences of annotation based on the Blastp, Blastx, KEGG, GO, EggNOG, and Pfam databases.

To identify the putative function of the assembled transcripts of *Cucumis sativus* L., sequence homology searches against the UniprotKB/SwissProt database using BLASTx and BLASTp displayed that 29,077 (81.42%) nucleotide sequences and 26,883 (75.28%) protein sequences were aligned (Supplementary Materials Table S3). Functional annotation of transcripts revealed that most of them (76%) have homologs in *Arabidopsis thaliana* (Figure 5B). Searching against the Pfam database resulted in the identification of 65,002 probable domains in the *C. sativus* transcriptome, of which 4047 were classified into unique domain groups. The most abundant domains were connected with the pentatricopeptide repeat (PPR) motif (PF01535.19, PF13041.5, PF13812.5, and PF12854.6). Only in 2285 and 6924 signal peptide transcripts and transmembrane regions were predicted with SignalP and TmHMM, respectively. A total of 15,623 were annotated in at least one database (Figure 5C).

Gene ontology (GO) functional analysis was performed to classify the assembled transcripts into three functional categories, i.e., biological processes (BP), molecular functions (MF), and cellular components (CC). In total, 27,573 (77.11%) out of all annotated transcripts were predicted with 7699, 4292, and 2517 gene ontology terms in BP, MF, and CC, respectively (Figure 6). The highly represented terms in all GO categories were presented in Figure 6A.

A total of 391 KEGG pathways were identified for 25,590 (71.7%) transcripts (Supplementary Materials Data S1). The annotated pathways were categorized into six main groups i.e., 'organism systems', 'cellular processes', 'environmental information process-

ing', 'genetic information processing', and 'metabolism' (Figure 6B). The highest number of transcripts (387) was assigned to 'plant hormone signal transduction pathway' [ko04075] in the 'environmental information processing' category. In the 'metabolism' category, the most dominant pathways were 'glycerophospholipid metabolism' [ko00564] (189 transcripts), 'amino sugar and nucleotide sugar metabolism' [ko00520] (184 transcripts), and 'purine metabolism' [ko00230] (175 transcripts), involved in 'lipid, carbohydrate, and nucleotide metabolism', respectively.

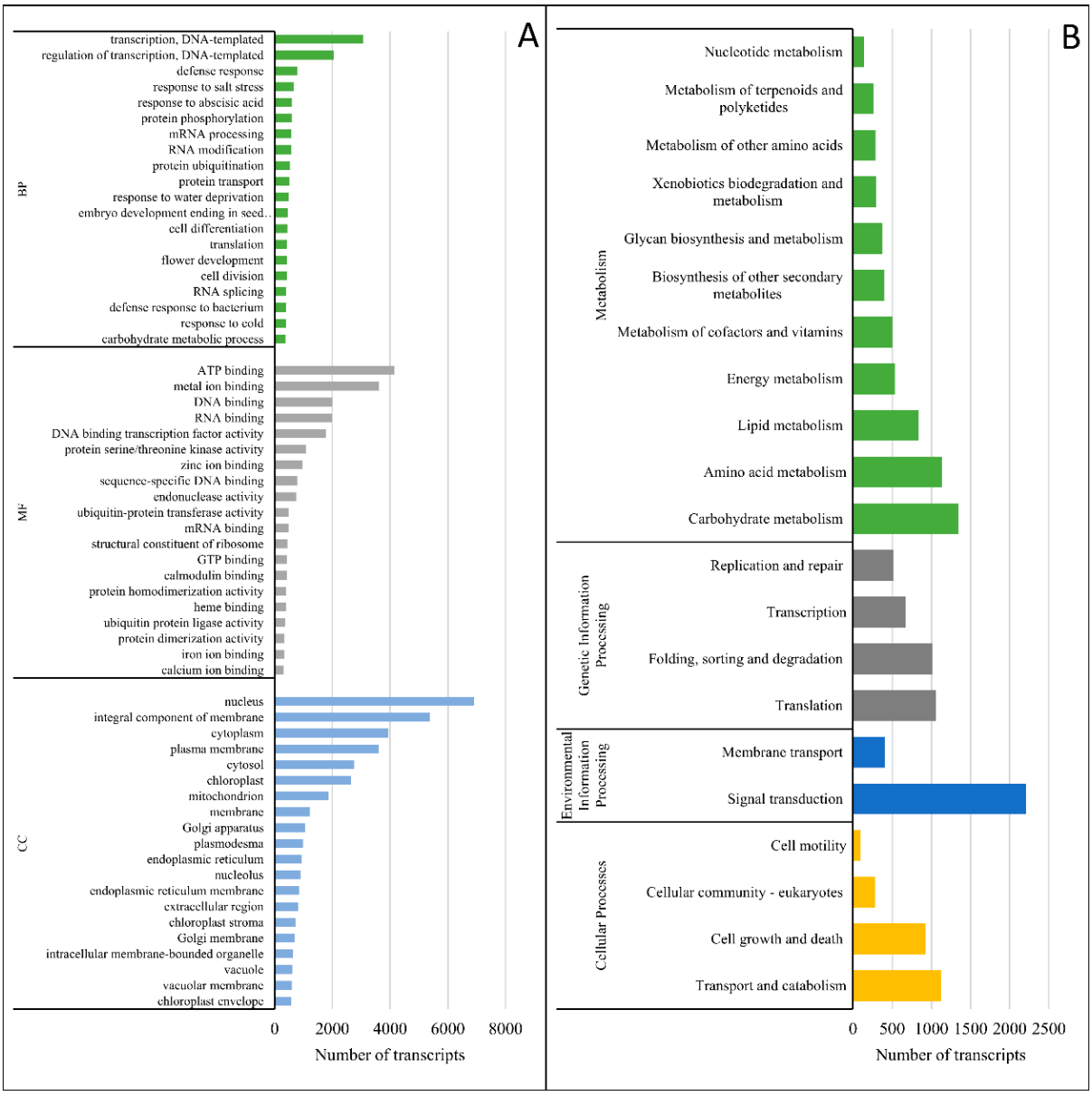


Figure 6. (**A**) Distribution of *C. sativus* transcripts in the top 20 gene ontology (GO) categories in biological processes (BP), molecular functions (MF), and cellular components (CC). (**B**) Pathway assignment was based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Classification was based on organismal system categories, cellular process categories, environmental information processing categories, genetic information processing categories, and metabolism categories.

3.4. Identification of Differentially Expressed Genes (DEGs)

In WL-T DH2, it was found that, under a single waterlogging treatment (1xH), 5957 genes were differently expressed, compared with untreated plants (Ctrl), including approximately 57% of the genes (3373) characterized by down-regulated expression and

43% (2584) of the genes showing over-expression (Table 2). After a 14-day recovery period (Rec), the number of DEGs decreased more than nine-fold, i.e., 654 DEGs were identified, of which 355 were overexpressed and 299 were diminished in expression compared to Ctrl. In plants treated a second time by the stress (2xH), 5007 significant DEGs were detected compared with the unstressed plants (Ctrl), of which 46% (2310) were positively expressed.

Table 2. Numbers of differentially expressed genes (DEGs).

Regulation	WL-T DH2				WL-S DH4			
	1xH vs. Ctrl	Rec vs. Ctrl	2xH vs. Ctrl	1xH vs. 2xH	1xH vs. Ctrl	Rec vs. Ctrl	2xH vs. Ctrl	1xH vs. 2xH
↑	2584 (493) *	355 (66)	2310 (474)	78 (13)	4211 (948)	892 (164)	5453 (1120)	1649 (355)
↓	3373 (560)	299 (51)	2697 (473)	348 (71)	4716 (809)	985 (249)	6166 (1112)	1196 (238)
Total	5957 (1053)	654 (117)	5007 (947)	426 (84)	8927 (1757)	1,877 (413)	11,619 (2232)	2845 (593)

* Number of differentially expressed genes for which FDR < 0.05. Numbers in brackets correspond to number of functionally uncharacterized and unannotated.

In the case of the WL-S DH4 accession, as a result of the first waterlogging stress (1xH), the number of genes with differential expression was 33% higher compared with WL-T DH2 i.e., 8927 DEGs were identified (Table 2). After the regeneration period (Rec) of waterlogged WL-S DH4 plants, the number of DEGs was reduced by nearly five-fold compared to 1xH plants (1877), of which 892 DEGs were up-regulated and 985 were down-regulated. The second stress (2xH) resulted in a further increase in the number of DEGs compared with unstressed plants (Ctrl), i.e., 11,619 DEGs were identified in total, which was the largest number of identified DEGs among the combinations. A list of all identified DEGs for all comparisons are presented in Supplementary Materials Data S2–S9.

3.5. Differential Response to Waterlogging of Primed and Non-Primed WL-T and WL-S Cucumbers

In this study, we wanted to examine if pre-treatment (priming) with waterlogging stress in two cucumber accessions with different stress tolerance would have an impact on the transcriptomic response to repeated treatment with waterlogging stress.

To identify the biological pathways activated in plants treated once (1xH) and twice (2xH) with hypoxic stress in WL-T DH2 and WL-S DH4 cucumbers, DEGs were mapped to the reference canonical pathways in KEGG (Figure 7). The common and specific pathways activated in both stress treatment i.e., 1xH and 2xH were indicated. In WL-T DH2 among specific biological pathways regulated in plants treated once with hypoxia (1xH), were activated i.e., 'basal transcription factors', 'RNA transport', 'endocytosis', 'spliceosome', and 'ribosome biogenesis in eucaryotes' assigned as activated pathways, whereas 'cutin, suberine, and wax biosynthesis' was suppressed (Figure 7A). The unique pathways in plants exposed to hypoxia stress for the second time (2xH) were also specified (Figure 7B). In this case, DEGs were assigned to the activation of 'riboflavin metabolism', 'propanoate metabolism', 'zeatin biosynthesis', 'valine, leucine and isoleucine degradation', 'biosynthesis of secondary metabolites', and suppression of 'diterpenoid biosynthesis'.

In case of the WL-S DH4 accession, the specific pathways activated under hypoxia in plants after 7 days of waterlogging treatment (1xH) were: 'circadian rhythm—plant', 'basal transcription factors', 'riboflavin metabolism', whereas 'glycosphingolipid biosynthesis—globo and isoglobo series' was suppressed (Figure 7C). In plants waterlogged again after 14 days of recovery (2xH), we found specific activated pathways connected with 'mRNA surveillance pathway', 'autophagy—other' and 'valine leucine and isoleucine degradation' (Figure 7D). KEGG pathway analysis revealed that WL-S DH4 strongly activated the biosynthesis of secondary metabolites in response to 7 days of waterlogging (1xH), whereas

this was not detected in the WL-T DH2 accession. Biosynthesis of secondary metabolites was activated only after the second treatment (2xH) in the WL tolerant cucumber (DH2).

In the 1xH WL-T DH2 plant group, the classification of DEGs into the specific metabolic pathways using the MapMan tool resulted in the 2998 DEGs mapped, of which 521 were visible in the metabolism overview scheme (Supplementary Materials Figure S2A,B). In contrast, in the 2xH plant group, 1629 DEGs were mapped and 297 DEGs were visible. In the DH4 cucumber, 2654 DEGs identified for 1xH were mapped, of which 489 DEGs were visible on the scheme, whereas in the 2xH plant group, 6341 DEGs were mapped, of which 1109 DEGs were assigned (Supplementary Materials Figure S2C,D).

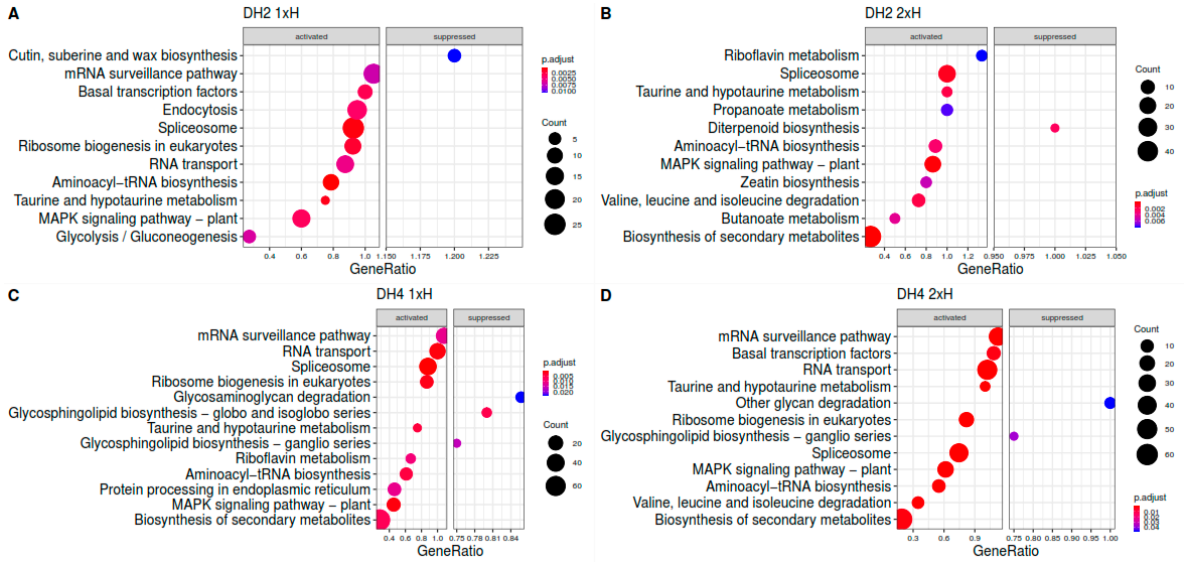


Figure 7. Scatter plot for the KEGG enrichment results obtained for WL-T DH2 (**A**,**B**) and WL-S (**C**,**D**) plants treated once (1xH) and twice (2xH) with waterlogging in comparison to untreated plants. The number of core genes ("count") divided by the total number of genes is the gene ratio. The sizes of the dots represent the number of core genes, and the color indicates the adjusted *p*-value. Only pathways with *p*-values < 0.05 were eligible for enriched biological processes.

DEGs identified in the 1xH and 2xH groups of both cucumber lines, involved in hormone metabolism, were mapped to the biosynthesis pathways associated with IAA, ABA, ethylene, cytokinins, and jasmonates. In WL-T DH2, the number of mapped genes was higher in plants treated once (1xH) in comparison to plants treated twice (2xH) (Supplementary Materials Figure S3A,B), whereas in WL-S DH4, the tendency was reverse, as a higher number of mapped genes involved in hormone metabolism were noted in plants waterlogged twice (2xH) (Supplementary Materials Figure S3C,D). The highest number of DEGs potentially involved in tolerance was assigned to the regulation of ABA and jasmonates.

Among all DEGs, we identified commonly regulated DEGs with 1xH and 2xH treatment and simultaneous statistically differential expression level between 1xH and 2xH. For this purpose, we compared DEGs in plants waterlogged once (1xH) and plants exposed twice to that stress (2xH) in comparison to control conditions (Ctrl) and also between them (Figure 8A,B). We identified 4079 and 7841 DEGs commonly expressed in 1xH and 2xH in WL-S DH2 and WL-T DH4, respectively. Additionally, we found that the common DEGs had strongly correlated expression patterns in 1xH and 2xH because the correlation coefficients was 0.96 and 0.94 for DH2 and DH4, respectively (Figure 8C,D). However, among the DEGs commonly expressed in 1xH and 2xH, we selected 132 and 1302 differentially expressed genes between 1xH and 2xH in WL-T DH2 and WL-S DH4, respectively (Figure 8E,F). We found 3 and 34 DEGs with opposite expression patterns in 1xH and 2xH in WL-T DH2 and WL-S DH4, respectively. In WL-T DH2, these DEGs were annotated as *α-amylase/subtilisin inhibitor* (XLOC_003864), *β-amyrin 11-oxidase* (XLOC_006633),

probable isoaspartyl peptidase/L-asparaginase 2 (XLOC_00945), revealing down-regulation in 1xH ($log_2FC = -2.96$, $log_2FC = -1.37$, $log_2FC = -1.30$, respectively) and up-regulation in 2xH ($log_2FC = 2.58$, $log_2FC = 1.54$, $log_2FC = 1.68$, respectively) (Supplementary Materials Table S4).

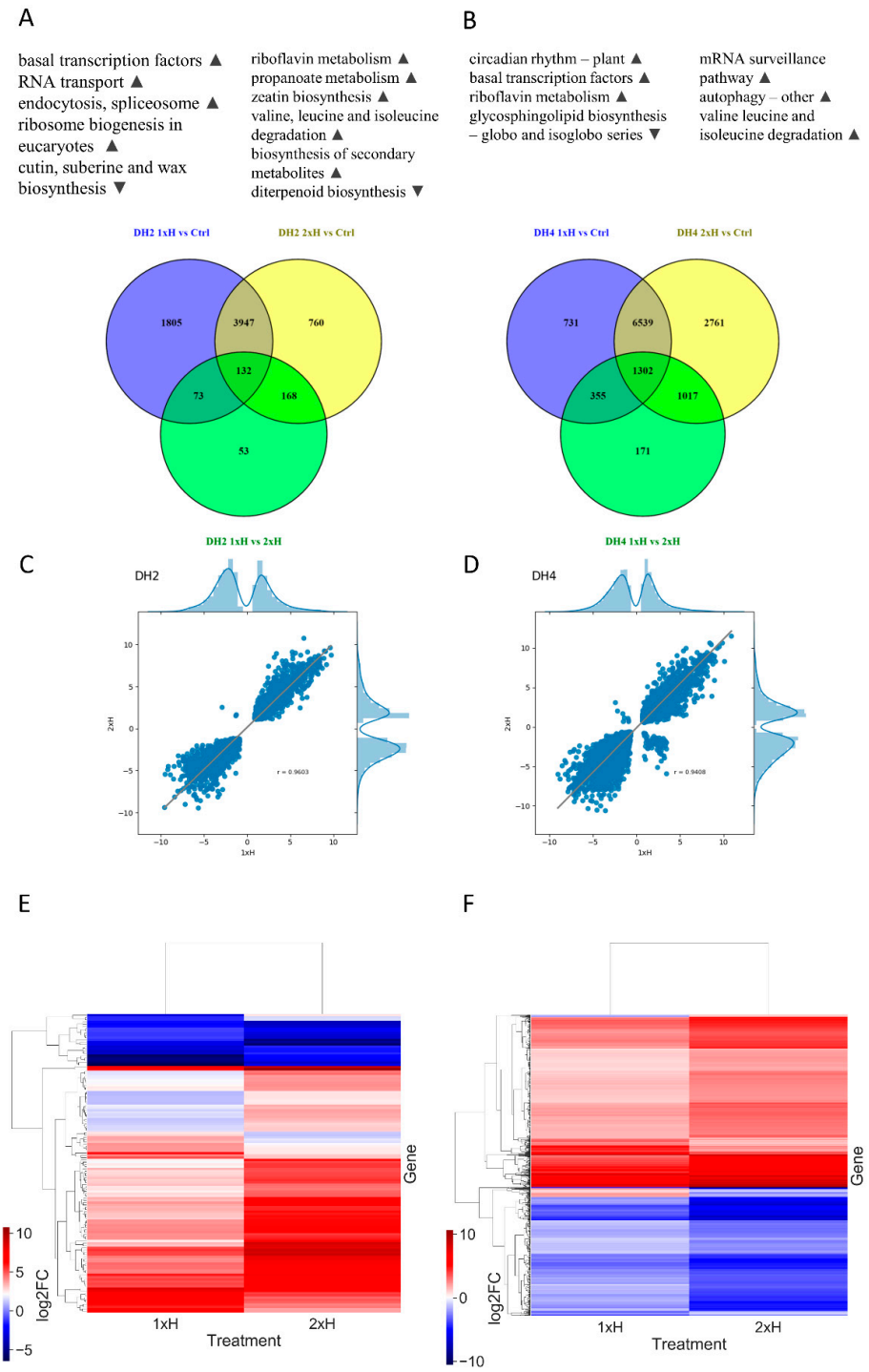


Figure 8. Number of differentially expressed genes (DEGs) specifically and commonly expressed in plants exposed to stress once (1xH) and twice (2xH) in the WL-T DH2 (**A**) and WL-S DH4 (**B**) accessions. Above the Venn diagrams, specific pathways for 1xH and 2xH treatment are listed. ▲ indicates up-regulation of pathway, ▼ indicates down-regulation of pathway. The correlation coefficient between 4079 and 7841 DEGs identified in plants treated once (1xH) and twice (2xH) in WL-T DH2 (**C**) and WL-S DH4 (**D**) is shown. Axes consist of Fold Change representing as ratio Treatment/Control. The heat map shows statistically significant DEGs commonly expressed in 1xH and 2xH in WL-T DH2 (**E**) and WL-S DH4 (**F**).

In the case of the WL sensitive accession (DH4), five DEGs were down-regulated with a single waterlogging treatment (1xH), but up-regulated after second waterlogging treatment (2xH); these included *sugar transporter ERD6-like 16* (XLOC_014798), *calmodulin-like protein 8* (XLOC_002728), and *protein SMAX1-LIKE 4* (XLOC_000765). These DEGs are involved in biological processes such as 'hexose transmembrane transport' (GO:0035428), 'detection of calcium ion' (GO:0005513), and 'carbohydrate homeostasis' (GO:0033500) and may be involved in acquiring tolerance to waterlogging in cucumber. The reverse expression pattern was noted in 29 DEGs, where among others, up-regulation of DEGs after 1xH and down-regulation after a second treatment (2xH) were noted for *cinnamoyl-CoA reductase 1* (XLOC_014021), *calcium-binding protein PBP1* (XLOC_014985), *MLP-like protein 328* (XLOC_011942), and *probable glycosyl transferase* (XLOC_003800). These DEGs were assigned to the following biological processes: 'lignin biosynthetic process' (GO:0009809), 'response to auxin' (GO:0009733), 'response to cytokinin' (GO:0009735), and 'cell wall organization' (GO:0071555), respectively. Moreover, 14 of 34 DEGs were annotated as unknown (Supplementary Materials Table S4), but they were strongly regulated by waterlogging and may play important roles in the response to oxygen deprivation. These genes with opposite expression levels allowed us to distinguish cucumber plants treated once (1xH) from plants treated twice with waterlogging (2xH).

Since accession DH2 is considered to be WL tolerant, we wanted to find DEGs uniquely regulated in WL-T after 1xH and in WL-S after the second exposure to stress (2xH) in order to indicate DEGs involved in enhanced tolerance to oxygen deprivation. As result, we identified 305 DEGs exclusively regulated both in the WL-T DH2 cucumber once waterlogged (1xH) in comparison to control plants and as well as in WL-S DH4 plants after the second stress treatment (2xH) (Figure 9). We found, among other DEGs, that *GDSL esterase/lipase*, *aspartate aminotransferase*, *mitochondrial*, *glutamate decarboxylase 3*, *triosephosphate isomerase*, *cytosolic*, *sucrose synthase*, and *expansin-like A1* were strongly up-regulated under hypoxic conditions in tolerant plants (WL-T) after a single waterlogging (1xH) and in the WL sensitive accession after the second treatment (2xH), meaning that the WL-S accession after the second treatment induced the expression of genes potentially connected with tolerance to oxygen deprivation (Supplementary Materials Data S10).

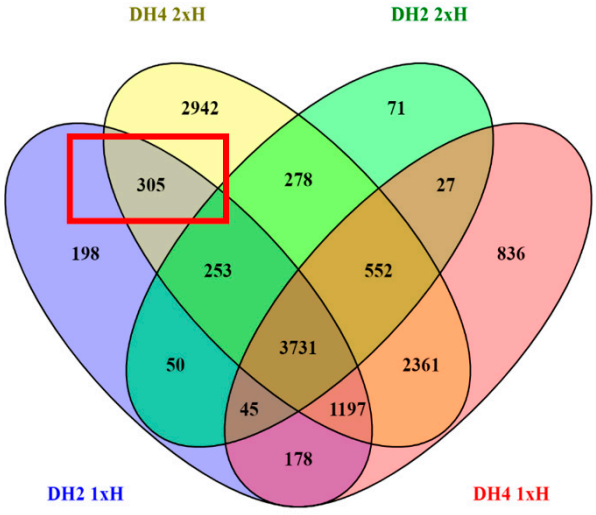


Figure 9. Venn diagram displaying common and specific DEGs regulated under 1xH and 2xH treatment in comparison to control conditions.

3.6. DEGs Determined in WL-T DH2 and WL-S DH4 Cucumbers in Recovery

To determine pathways and genes regulated in long-term recovery period after hypoxic stress in the WL-T DH2 and WL-S DH4 accessions, we analyzed the comparison Rec vs. Ctrl in both DH lines. Gene ontology (GO) enrichment of the DEGs identified in

WL-T DH2 and WL-S DH4 were presented as Supplementary Materials Tables S5 and S6, respectively.

Considering common BP and MF terms in WL-T (DH2) and WL-S (DH4), 21 terms in the BP category and 20 terms in the MF category were indicated, respectively (Figure 10A,B). For both the BP and MF terms, the most differentiating ones were processes connected with carbohydrate metabolism, i.e., carbohydrate catabolic process (GO:0016052) and carbohydrate binding (GO:0030246), which were significantly altered in WL-S DH4 (Figure 10B). Among the pool of unique DEGs involved in carbohydrate metabolism in WL-S DH4 were *probable pectate lyase 22*, *β-amylase 3*, *multiple inositol polyphosphate phosphatase 1*, *putative glucose-6-phosphate 1-epimerase*, *β-galactosidase 3*, *endoglucanase 6*, *naringenin, 2-oxoglutarate 3-dioxygenase*, *ACC oxidase 1*, and *ACC oxidase 3* and two DEGs in WL-T DH2, i.e., *pyruvate dehydrogenase E1 component subunit α* and *F-box protein PP2-B15*.

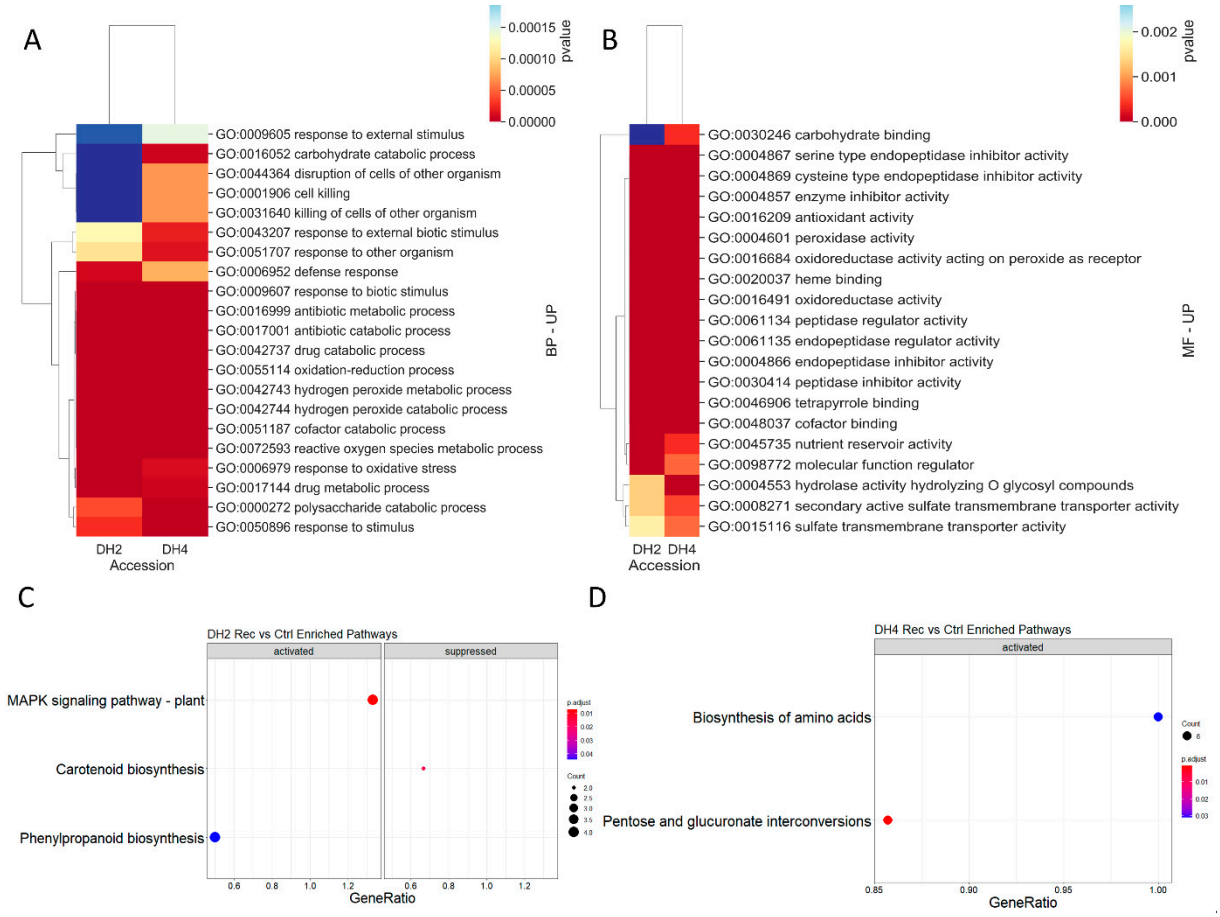


Figure 10. GO terms commonly enriched into (**A**) biological processes and (**B**) molecular functions after the recovery period in the DH2 and DH4 lines. Scatter plot for KEGG enrichment results obtained for plants after the recovery period in comparison to untreated plants in the DH2 (**C**) and DH4 (**D**) lines. The number of core genes ("count") divided by the total number of genes is the gene ratio. The sizes of the dots represent the number of core genes, and the color indicates the adjusted *p*-value. Only pathways with *p*-values < 0.05 were eligible for enriched biological processes.

For DEGs identified in the WL-T DH2 accession, KEGG analysis indicated pathways related to 'MAPK signaling pathway—plant', 'phenylpropanoid biosynthesis' and 'carotenoid biosynthesis' (Figure 10C). In the WL-S DH4 accession, two significantly activated pathways were identified, i.e., 'pentose and glucoronate interconversion' and 'biosynthesis of amino acids' (Figure 10D).

3.7. qRT-PCR Analysis

The results show that the expression level of genes, i.e., *alcohol dehydrogenase* (*adh*), 1-aminocyclopropane-1-carboxylate oxidase (*aco*), and *long-chain acyl-CoA synthases 6* (*lacs6*) involved in response to hypoxia were differentially regulated between the WL-T and WL-S cucumber DH lines. The relative expression level of the *adh* gene was approximately two-fold higher in WL-S DH4 plants under waterlogging stress than in WL-T DH2 plants (Figure 11A,B). In WL-T DH2, after the second waterlogging (2xH), *adh* gene expression increased from day 1 to 3 (22–24 days of the experiment), then decreased, while in WL-S DH4, the *adh* gene was up-regulated to the same level throughout the 7 days of stress.

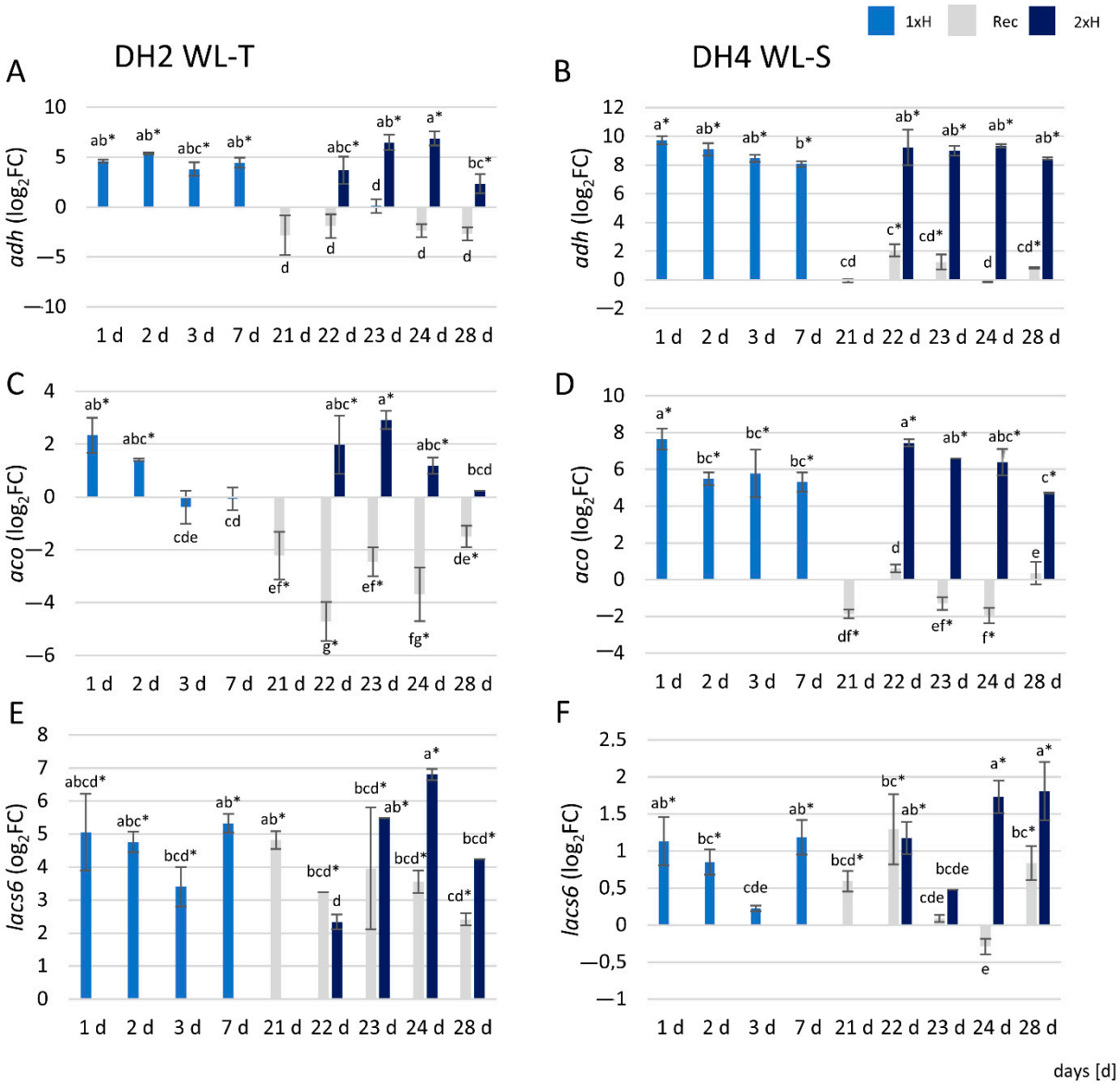


Figure 11. Expression profiles of the *adh* (**A**,**B**), *aco* (**C**,**D**), and *lacs6* (**E**,**F**) genes in WL-T DH2 and WL-S DH4 cucumber accessions under waterlogging stress, respectively. Data are expressed as the mean ± SD (standard deviation) of three independent biological replicates and three technical replications with $p < 0.05$ (Tukey's post-hoc test). Asterisks indicate a significant difference vs. control plants. The same letters indicate no statistical differences between treatments and time-points (days).

The expression level of the *aco* gene was significantly increased after 1 d of waterlogging treatment in the 1xH group of both cucumber lines; however, in WL-S DH4, the

expression level was 2.5 times higher than in WL-T DH2. Up-regulation of the *aco* gene in WL-T DH2 was detected at 1 and 2 d after the first (1xH) and second (2xH) waterlogging treatments. Subsequently, expression declined, and at 7 d was comparable to control. In contrast, in WL-S DH4, up-regulation of the *aco* gene was maintained at the same level during the 7 days of the first (1xH) and second (2xH) waterlogging treatments. In recovered plants (Rec), the *aco* gene was down-regulated in both lines (Figure 11C,D).

Waterlogging treatment enhanced the expression of the *lacs6* gene just 1 d after stress induction in both cucumber lines; however, in WL-T DH2, up-regulation of the *lacs6* gene was meaningful, compared to the expression level detected in WT-S DH4. After a second waterlogging treatment (2xH), *lacs6* was positively regulated at 1 d in WL-S DH4, whereas in WL-T DH2, up-regulation occurred one day later, i.e., after 2 days of stress. In WL-T DH2, *lacs6* was also up-regulated in recovered plants (Rec) of both cucumber accessions (Figure 11E,F).

4. Discussion

Hypoxic stress in plants provokes changes on the morphological, physiological, and molecular levels. In this study, we compared responses to long-term waterlogging and recovery on the morphological and molecular levels in two cucumber accessions with divergent tolerance.

4.1. Morphological Changes in Response to Waterlogging Stress

In terms of morphological changes, discrepancies were observed between cucumber accessions. In WL-T DH2, no effect of hypoxic stress on plant height or the number of leaves was demonstrated, which in turn was observed in WL-S DH4, where hypoxic stress slowed down plant growth and reduced the number of leaves. In WL-T cucumbers, slower plant growth was first observed in the recovery period compared to control plants. The lack of difference in plant height in the WL-T accession in the context of hypoxia stress may be related to its tolerance. It has been shown that, in tolerant accessions, the height of stress-treated plants does not change compared to control plants, while in sensitive cucumber plants growth is slower [56,57]. Biomass accumulation in upper part of cucumber plants did not change under first waterlogging treatment in both DH lines. It was shown that under 8 days of waterlogging, biomass accumulation (DM:FM) was higher in treated cucumber plants, however the level of tolerance of tested cucumber was not provided. We carried out our analysis of a set of accessions with evidenced tolerance level [10], so based on our results, DM:FM has no impact on the tolerance level to hypoxia stress in tested lines. The second waterlogging (2xH) resulted in slower plant growth and biomass increase compared to the control plants, which may be due to the fact that, plants were focused on processes related to overcoming stress and not on the growth.

Differences in the response to stress were observed in the mass of the roots formed by cucumber plants. The increased ARN (hypocotyl-derived adventitious root number) that developed under hypoxic stress is considered to be a trait associated with tolerance and adaptation to limited oxygen access, as it facilitates gas exchange between the aerial zone and waterlogged soil [8,58,59]. Qi et al. [60] reported that tolerant and sensitive accessions were selected based on the ARN, where a large number of adventitious roots was observed in the tolerant accession, and a small number of AR was seen in the sensitive accession. These observations also correlate with the results we obtained, where WL-T DH2 produced a significantly greater mass of AR after 7 days of stress compared to the WL-S DH4. Enhanced development of ARN as an adaptation to waterlogging has also been observed in barley and tomato [56,61].

4.2. Changes at the Transcriptomic Level

The literature provides information on variations in the number of regulated transcripts under oxygen deprivation stress in genotypes with different levels of tolerance [13,62]. Kreuzwieser et al. [63] observed that the number of transcripts increased in the case of

gray poplar genotypes characterized by increased hypoxia sensitivity. Here, the accession indicated as tolerant (DH2) demonstrated fewer transcripts with differential expression than the sensitive accession (DH4) under long-term waterlogging. The same trend was followed in cucumber by Xu et al. [17]; however, there the waterlogging stress lasted only 2 days. There are no transcriptomic data in the literature to compare cucumber accessions with contrasting responses to hypoxia. We also obtained a variable number of DEGs in plants that were treated again with hypoxic stress. In the case of the tolerant accession, the number of DEGs decreased after recurrent stress, while in the sensitive accession, the number of regulated transcripts increased. This may indicate that first waterlogging treatment enhanced tolerance to recurring waterlogging stress in the sensitive accession. An increased number of DEGs in primed plants, compared to non-primed plants, was also observed in rice, where salt shock was used as the priming factor [64] and in radish, where cold priming was applied [65]. To the best of our knowledge, there is no information on the differential number of regulated genes between plants treated once with hypoxic stress and plants treated again with stress, thus these results are novel and unique in the area of waterlogging tolerance in cucumber. Additionally, differences in the response to oxygen deprivation between DH2 and DH4 were observed at the transcriptomic level.

4.3. Effects of Waterlogging Stress Priming on the WL-T and WL-S Cucumber Accessions

Both cucumber accessions, under long-term hypoxic stress, enhanced pathways connected with translation i.e., 'aminoacyl-tRNA biosynthesis' and 'RNA transport', transcription i.e., 'basal transcription factors' and 'spliceosome', and signal transduction by activating 'MAPK signaling pathway—plant'. In the WL tolerant cucumber, the 'alanine, aspartate and glutamate metabolism' pathway was activated, whereas in the WL sensitive cucumber, 'biosynthesis of secondary metabolites' was observed. The activation of 'alanine, aspartate, and glutamate metabolism' was also reported in cucumber hypocotyls of the WL-S cucumber accession [59]. Biosynthesis of secondary metabolites was also activated in the roots of rapeseed but, in contrast to our results, in the tolerant accession [13]. These differences could be explained manly due to the duration of exposure to waterlogging and the tissue that was analyzed. We can assume that cucumber plants cope with stress conditions by activating different pathways depending on exposure duration. There is no information on the use of RNA-Seq to find differences between accessions with contrasting hypoxia responses in cucumber at the root system level, so undoubtedly these results will provide sufficient knowledge in this area.

According to these results, in the WL tolerant accession after the second exposure to waterlogging, unique pathways were activated such as: 'riboflavin metabolism', 'propanoate metabolism', 'zeatin biosynthesis', and 'valine, leucine, and isoleucine degradation' related to amino acid metabolism. These pathways were also activated in the WL sensitive cucumber primed by 7 days waterlogging. To our knowledge, data associated with the response to a second treatment have not been reported so far. We can state that the pathway 'valine, leucine, and isoleucine degradation' is activated after a second exposure to waterlogging in cucumber. Additionally, in the WL sensitive accession, the 'mRNA surveillance pathway' was also activated in plants treated twice with waterlogging.

We also found differentially expressed genes in relation to control presenting contrasting expression levels with single and repeated waterlogging treatment. In the WL-T accession, the expression of *α-amylase/subtilisin inhibitor*, involved in starch and sucrose metabolism, was inhibited after a single waterlogging treatment, whereas after the second treatment, it was up-regulated. In barley, up-regulation of this gene has been reported in tolerant and moderately tolerant genotypes, but this observation was detected after 3 and 5 days of waterlogging. These contrasting results point out differences in the response to hypoxic stress between species [66]. In the WL-S cucumber, a similar expression pattern was demonstrated for *sugar transporter ERD6-like 16* and *calmodulin-like protein 8* genes. In radish, enhanced expression of the *sugar transporter ERD6-like* gene, considered to be one of the genes interacting with MaRAP2-4, provides abiotic stresses tolerance [67]. Since

the WL-S accession revealed up-regulation of this gene after the second treatment (2xH), it could suggest that waterlogging acted as a priming factor in acquiring tolerance to waterlogging stress in the WL-S accession. Calmodulin-like proteins (CMLs) are sensors of Ca^{2+} and take part in calcium signaling [68]. It has been reported that CMLs may be involved in aerenchyma formation, allowing plants to perform gas exchange between roots and shoots [69].

4.4. Genes Potentially Involved in Tolerance to Long-Term Waterlogging in Cucumber

We indicated the genes potentially involved in acquiring tolerance to waterlogging stress in cucumber. These genes were uniquely up-regulated after a single waterlogging treatment in the WL-T accession and in the WL-S accession after the second exposure to stress. We observed strong up-regulation of *GDSL esterase/lipase* gene in our results. Enhanced expression of *GDSL esterase/lipase* gene and high accumulation of GDSL esterase/lipase protein were also observed in the tolerant cucumber line Zaoer-N in hypocotyls and roots, respectively [17,59]. These results suggest enhanced lipid catabolism in cucumbers with tolerance to hypoxic stress. As a result of *GDSL esterase/lipase* activity, glycerol and free fatty acids are produced. Glycerol may be used for carbon and energy supply and for adventitious roots development, as we confirmed here as being characteristic of the WL-T DH2 accession. Free fatty acids, due to the loss of cell walls during hypoxia, may provide components for newly generated cells.

Aspartate aminotransferase (AspAt) transfers N from aspartate to glutamate, which is a substrate of GABA synthesis, where GAD (glutamate decarboxylase) is involved [70]. Aspartate aminotransferase and glutamate decarboxylase also participate in amino acid metabolism and serves as regulators of cytoplasmic pH as it becomes lower during hypoxic stress, mostly due to enhanced lactic acid production [71]. In soybean hypocotyls, the *Aspartate aminotransferase* gene is up-regulated under flooding conditions [72]. Up-regulation of *AspAt* was also detected in the roots of a tolerant maize genotype [4]. In our study, up-regulation was demonstrated in the WL-T DH2 accession after a single waterlogging treatment, and as well as in the WL-S accession after subsequent stress induction. These results allow us to claim that the first stress treatment in the WL-S accession enhanced tolerance to oxygen deprivation.

The *glutamate decarboxylase* (*GAD*) gene is activated under oxygen deprivation and regulates the production of γ-amino butyric acid (GABA) [73]. GABA, by activating a number of genes, participates in plant adaptation to hypoxic stress caused by flooding [74]. Enhanced expression of *GAD*, exclusively in plants of the WL-T line cucumber after single hypoxia treatment and in plants of the WL-S line after the second treatment, may indicate that the WL-S cucumber line acquires tolerance to oxygen deprivation.

The *sucrose synthase* (*SuSy*) gene encodes an enzyme that participates in low oxygen level tolerance [75,76]. The role of *Susy* is the hydrolysis of sucrose to fructose and UDP-glucose in order to drive ATP production by glycolysis, which is important in oxygen deprivation tolerance [77]. Up-regulation of *SuSy* was determined in WL-tolerant cucumber [52], maize [78]), and barley [66] and it was also detected in our study, so it may be assumed that *SuSy* is involved in tolerance to long-term waterlogging.

In addition to the *SuSy* gene, we also detected up-regulation of *Triosephosphate isomerase, cytosolic* (*TPI*), an important glycolytic enzyme [79]. It has been reported that *TPI* is up-regulated in the roots of a WL-tolerant accession of maize [78], and our results confirm this tendency.

Expansins are responsible for alleviation of cell walls, resulting in its extension [80]. Increased expression has been observed under the influence of abiotic stress, such as drought [81], salinity [82], waterlogging [83,84], as well as also under the influence of low pH (~4.8). It was reported that increased development of root hair was observed in the RhEXPA4 transgenic line of *Arabidopsis* [85], which may correlate with the increased number of side roots that are produced under the limited oxygen access in tolerant plants. In maize, up-regulation of the *expansin-like A1* gene was found in the roots of a tolerant

accession under waterlogging stress [84] as in our work. Higher expression of expansin genes contributes to the mitigation of hypoxic stress in root zones.

4.5. DEGs Determined in WL-S and WL-T Cucumbers in Recovery

The recovery period following the removal of hypoxic stress refers to reoxygenation [86]; however, the shift to normoxic conditions generates additional stresses, i.e., oxidative stress and dehydration due to damaged roots. For this reason, waterlogging is described as a sequential stress where the waterlogging and recovery periods pose distinct stressors. The ability to acclimate to both waterlogging and recovery is crucial for the determination of tolerance to hypoxic stress in plants [19,87]. The molecular mechanisms of the response to post-waterlogging reoxygenation in plants are poorly understood, and they have been investigated mainly within a short time frame after stress. In the recovery period, oxidative stress has been shown to be induced in plants because of dehydration caused by dysfunction of the root system and consequent generation of ROS, ABA, and ethylene [19,88]. Here, we exploited two cucumber accessions differing in waterlogging tolerance [10] and we compared the expression profiles of genes in plants after 14 days of recovery compared to control plants. Differential recovery between the WL-T DH2 and WL-S DH4 accessions was related to the activity of genes related to carbohydrate metabolic processes, which were significantly up-regulated in the WL-S accession. Among the pool of unique DEGs involved in carbohydrate metabolism in WL-S DH4 were the *probable pectate lyase 22*, *β-galactosidase 3*, and *endoglucanase 6* genes, encoding proteins involved in the degradation structural polysaccharides in plant cell walls. These genes were down-regulated under flooding stress, suggesting that the reduced accumulation of encoded proteins could lead to arrested root growth and the suppression of lateral root formation [89]. After 14 days of recovery, we observed increased expression of these genes only in the waterlogging sensitive cucumber, suggesting more intensive cell wall reconstruction processes and growth of the root system in comparison to WL-T cucumbers. The expression levels of the *ACC oxidase* 1 and *ACC oxidase* 3 genes, involved in the conversion of 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene, were also higher in the WL-S DH4 accession. These results suggest that long-term recovery in the WL-S cucumber was associated with ethylene biosynthesis at the transcriptional level. It has been reported that ethylene is synthesized during reoxygenation in submerged cucumber roots [90]. Tsai et al. [91] found that the expression levels of several *aminocyclopropane-1-carboxylic acid (ACC) synthase* and *ACC oxidase* genes were enhanced during the reoxygenation stage in *Arabidopsis*. They showed that ethylene plays a beneficial role in recovery by maintaining the balance of hormone signaling, including ethylene, as well as ABA and JA (Jasmonic acid). In WL-S DH4, the *naringenin 2-oxoglutarate 3-dioxygenase* gene, encoding an enzyme catalyzing the 3-hydroxylation of (2*S*)-flavanones such as naringenin to dihydroflavonols [92], was activated. Up-regulation of flavonoid pathway-related genes has been observed in potato, rice, and *Reaumuria soongorica* after exposure to drought and UV stress [93–95]; collectively, these studies indicate that the flavonoid pathway is involved in stress tolerance.

After 14 days of recovery, in the waterlogging tolerant cucumber (DH2), increased expression of *pyruvate dehydrogenase E1 component subunit α* gene was reported. Under aerobic conditions, pyruvate is converted to acetyl-CoA by the pyruvate dehydrogenase enzymatic complex. If oxygen levels decrease, NADH accumulates and the pyruvate dehydrogenase complex unit is inhibited [96]. Interestingly, in our study, the highest expression of this gene was found in recovery compared to control and only in the WL-tolerant cucumber accession.

4.6. qRT-PCR Assay Revealed Differences Throughout Waterlogging Stress Duration

Increased expression of *alcohol dehydrogenase* (*adh*) is used as a molecular marker for the stress response to hypoxia in plants [97]. Higher levels of *adh* transcripts were noted in the WL-T DH2 cucumber. Plants intensively activate the alcoholic fermentation pathway in order to produce NAD^+ and maintain glycolysis under conditions of low oxygen

concentration. NAD^+ regeneration from NADH is considered to be the most important function of the alcoholic fermentation pathway under oxygen restricted conditions [98]. An increased expression level of *adh* under hypoxic stress was also demonstrated in the roots of radish [99], poplar [63], and the cucumber genotype Zaoer, which has a high level of tolerance to flooding stress [52]. Relative expression levels of *adh* obtained for both cucumber accessions by qRT-PCR correspond to RNA-Seq results.

Ethylene is a plant hormone known as a signaling and regulatory molecule in response to plant hypoxic stress [100]. It is synthetized from methionine, which is first converted to S-adenosylmethionine (AdoMet) by S-adenosylmethionine synthase, then converted to 1-aminocyclopropane-1-carboxylate (ACC) by ACC synthase (ACS) [101]. Ethylene synthesis requires oxygen at the level of 1-aminocyclopropane-1-carboxylate oxidase (*aco*), which catalyzes the final step in ethylene biosynthesis. Differences in the expression level of the *aco* gene in response to hypoxic stress were found in both cucumber accessions, demonstrating contrasting reactions to limited oxygen access, which indicates the different mechanisms related to the response to oxygen deprivation in the cucumber root system. Increased expression of the *aco* gene under hypoxic stress was also demonstrated by Qi et al. [52] in cucumber roots 4 hours after stress induction. *Aco* expression level determined by qRT-PCR in WL-S DH4 correlated with RNA-Seq results.

Long-chain acyl-CoA synthases (LACS) activate free fatty acids to acyl-CoA thioesters. This class of enzymes is involved in several fatty acid metabolic pathways, including phospholipid biosynthesis and β-fatty acid oxidation [102], which are essential for maintaining cell homeostasis [103]. Lipids are essential cellular components that not only provide the structural basis for cell membranes and energy for metabolic processes, but also serve as signals in plant responses to environmental signals [104]. In roots, the LACS gene product may be involved in the suberin and cuticle waxes biosynthesis [105]. The cutcle can change in response to various abiotic or biotic stresses [106]. It was found that the relative expression level of the *lacs 6* gene was significantly higher in the DH2 cucumber line, indicative of tolerance to hypoxic stress, which may suggest that the β-lipid oxidation process was more intense. This also correlates with the amount of ascorbic acid and glutathione determined in the tested cucumber lines (unpublished data), which are components of the antioxidant system in plants, protecting cells against the negative effects of reactive oxygen species (ROS). qRT-PCR revealed higher expression of *lacs6* in WL-S after 1xH and 2xH treatment than it was determined in the RNA-Seq approach, whereas in WL-S, results obtained by qRT-PCR correlated with RNA-Seq.

5. Conclusions

Nowadays, developing methods to improve tolerance to abiotic and biotic stresses is the main goal of breeders to reduce losses associated with climate change [107] in the context of food protection [31]. In this study, we obtained results supporting waterlogging as a priming factor against waterlogging stress in cucumber. The results obtained in this study provide new and unique information regarding the responses to long-term hypoxia in cucumber in terms of priming. However, a significant percentage of the identified DEGs were functionally unannotated and uncharacterized, but can be assigned to specific cucumber genes, transposable elements, or ncRNA that may be involved in post-transcriptional regulation. This aspect requires further investigation and will be examined in the future.

Supplementary Materials: The following are available online at https://www.mdpi.com/2073-4425/12/2/189/s1, Figure S1: Number of Differentially Expressed Genes identified by DESeq2 and edgeR in two cucumber DH lines under waterlogging stress, Figure S2: Pictorial representation of genes in WL-T DH2 and WL-S DH4 accessions associated with various MapMan functional metabolism categories under waterlogging stress, Figure S3: Pictorial representation of genes in WL-T DH2 (A, B) and WL-S DH4 (C, D) accessions associated with MapMan functional regulation overview under single (1xH) and repeated (2xH) waterlogging stress. Table S1: Genes data and primers used in the analysis, Table S2: Summary of RNA-Seq and mapping details, Table S3: Statistics of annotation results for *Cucumis sativus* L. transcripts, Table S4: Genes with opposite expression in

cucumber plants treated once (1xH) and twice (2xH) with waterlogging, Table S5: Gene ontology enrichment of DEGs identified in WL-T in Rec, Table S6: Gene ontology enrichment of DEGs identified in WL-S in Rec, Data S1: KEGG mapping of *Cucumis sativus* L. transcripts, Data S2: DEGs identified in comparison DH2 1xH vs. Ctrl, Data S3: DEGs identified in comparison DH2 Rec vs. Ctrl, Data S4: DEGs identified in comparison DH2 2xH vs. Ctrl, Data S5: DEGs identified in comparison DH2 1xH vs. 2xH, Data S6: DEGs identified in comparison DH4 1xH vs. Ctrl, Data S7: DEGs identified in comparison DH4 Rec vs. Ctrl, Data S8: DEGs identified in comparison DH4 2xH vs. Ctrl, Data S9: DEGs identified in comparison DH4 1xH vs. 2xH, Data S10: Common 305 DEGs identified in DH2 1xH vs. Ctrl and DH4 2xH vs. Ctrl.

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Waterlogging-Stress-Responsive LncRNAs, Their Regulatory Relationships with miRNAs and Target Genes in Cucumber (*Cucumis sativus* L.)

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Abstract: Low oxygen level is a phenomenon often occurring during the cucumber cultivation period. Genes involved in adaptations to stress can be regulated by non-coding RNA. The aim was the identification of long non-coding RNAs (lncRNAs) involved in the response to long-term waterlogging stress in two cucumber haploid lines, i.e., DH2 (waterlogging tolerant—WL-T) and DH4 (waterlogging sensitive—WL-S). Plants, at the juvenile stage, were waterlogged for 7 days (non-primed, 1xH), and after a 14-day recovery period, plants were stressed again for another 7 days (primed, 2xH). Roots were collected for high-throughput RNA sequencing. Implementation of the bioinformatic pipeline made it possible to determine specific lncRNAs for non-primed and primed plants of both accessions, highlighting differential responses to hypoxia stress. In total, 3738 lncRNA molecules were identified. The highest number (1476) of unique lncRNAs was determined for non-primed WL-S plants. Seventy-one lncRNAs were depicted as potentially being involved in acquiring tolerance to hypoxia in cucumber. Understanding the mechanism of gene regulation under long-term waterlogging by lncRNAs and their interactions with miRNAs provides sufficient information in terms of adaptation to the oxygen deprivation in cucumber. To the best of our knowledge, this is the first report concerning the role of lncRNAs in the regulation of long-term waterlogging tolerance by priming application in cucumber.

Keywords: cucumber; hypoxia; lncRNA; long-term waterlogging; miRNA; priming

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1. Introduction

Plants are constantly exposed to unfavourable environmental factors; therefore, a condition for their survival is the rapid activation of defence mechanisms and the ability to adapt to stressful conditions. Lowering the oxygen content below optimal conditions, referred to hypoxia, is a phenomenon that often occurs in the natural environment of plants [1,2]. Limited availability of oxygen in the root zone negatively affects the metabolism of the whole plant, impairing the growth and development [3]. Plants, however, have evolved adaptive mechanisms, causing changes at the molecular, biochemical and physiological levels, consequently leading to morphological changes that allow the transport of oxygen to the insufficiently oxygenated zones [4].

One of the ways to increase stress tolerance in plants is a priming mechanism, i.e., exposure of plants to stress conditions, leading to changes at the physiological and molecular levels, allowing plants to develop a more effective response when faced with another stress induction [5,6]. In our previous work, by priming application, we depicted six genes, i.e., GDSL esterase/lipase, aspartate aminotransferase (*AspAt*), glutamate decarboxylase (*GAD*), sucrose synthase (*SuSy*), triosephosphate isomerase, cytosolic (*TPI*) and expansin-like A1 (*EXP1*) involved in acquiring tolerance to long-term waterlogging in cucumber [7].

Regulation of those genes can be mediated by non-coding RNAs (ncRNAs) [8,9], which include, among others, micro RNAs (miRNAs), small interfering RNAs (siRNAs) and long non-coding RNAs (lncRNAs) [10].

miRNAs are the best known and most abundant short regulatory RNA molecules in plant and animal cells, with a typical length of 20 to 24 nucleotides. The biogenesis of plant miRNAs takes place almost entirely in the cell nucleus. The miRNA genes are first transcribed, most often by RNA polymerase II, generating primary miRNA transcripts (pri-miRNAs), which are then subjected to catalytic cleavage to produce the so-called pre-miRNA molecules, typically 50 to 100 nucleotides in length [11], forming characteristic secondary structures, dubbed hairpins or stem-loops. In the next stage, the mature miRNA is excised from the pre-miRNA and incorporated into the RISC (RNA-Induced Silencing Complex) silencing complex, which participates in the processes of gene expression regulation [12]. miRNAs control gene expression at the post-transcriptional level by inhibiting mRNA translation or cutting the transcript by the AGO1 protein [13] using extensive complementarity to the target sequence [14,15].

In plants, miRNAs regulate proper tissue differentiation, organ and the vascular system development [16]. The increased expression of miRNA data was also demonstrated in plants subjected to various stress factors, such as drought [17], salinity [18], heat [19], nutrient deficiency [20], or heavy metals [21], which indicate that miRNAs are also involved in the adaptation mechanisms to stressful conditions [22]. When it comes to hypoxia, studies concerning identification of miRNAs were conducted in *Arabidopsis thaliana* [23], *Zea mays* [24], *Populus tomentosa* [25], *Medicago sativa* [26], *Solanum habrochaites* [27] and *Cucumis sativus* in the context of adventitious roots formation [28].

LncRNAs represent another class of regulatory transcripts that participate in response to stresses in plants. They are defined as RNA molecules over 200 bp in length that are not translated into functional proteins. They are mainly located in the cell nucleus, in chromatin fractions, but also with a lower frequency in the cytosol [29]. LncRNAs are tissue-specific molecules with low expression levels and a low level of conservation between species [30].

LncRNAs have been classified according to their location in the genome and the function they play in cells. Taking into account their relative orientation towards proximal protein-coding genes, they can be defined as sense, antisense, bidirectional, intronic, and intergenic lncRNAs [31]. LncRNAs can act as enhancers or repressors of gene expression in either cis or trans regulation in a way [32]. They regulate gene activity on transcriptional, post-transcriptional or translational levels and by interactions with DNA, RNA and proteins [33,34].

LncRNAs are the least understood group of transcripts in the genomes of living organisms. Identification and determination of the functions of individual lncRNAs have so far been widely described, mainly in humans, but also in other animals [35–37]. In plants, they were only identified in a few species [38], which indicates the need to extend research in this area. The first work describing the function of lncRNA molecules in plants was published in 2004. The function of lncRNA, known as Enod40 in alfalfa, which is involved in the relocation of the nuclear RNA binding protein to the cytoplasm, was then determined [39]. Subsequent studies have shown that in plants, lncRNAs play a key role in the flowering and reproduction process [40], root organogenesis and seedling photomorphogenesis [41,42]. The lncRNAs expressed in response to biotic and abiotic stresses were also characterized [43–46].

Cucumber (*Cucumis sativus* L.) is an annual plant from the *Cucurbitaceae* family, characterized by a shallow root system. Throughout the vegetation, cucumber is exposed to several unfavourable environmental factors that lead to limited availability of oxygen [47,48]. One of them is excess water in the soil, which negatively affects the productivity of crops [49,50].

Many studies have demonstrated that the process of post-transcriptional regulation of gene expression with the participation of non-coding RNA molecules is crucial in response to environmental changes in plants [51,52], because it enables survival and activates

adaptive mechanisms to stress factors [53]. There is no information in the literature on the role of non-coding RNA molecules in the post-transcriptional regulation of genes induced in conditions of oxygen deficiency in the cucumber root zone; hence, it is important to broaden the knowledge in this field. Now, thanks to the rapid advances in deep transcriptome sequencing (RNA-Seq) technology and related bioinformatics methods, in silico tools are available to identify novel non-coding RNA molecules.

The aim of the study was to identify lncRNAs and miRNAs that participate in response to long-term waterlogging stress in cucumber accessions with confirmed diverse tolerance [7,54]. Additionally, an attempt to identify lncRNAs involved in acquiring tolerance to oxygen deprivation in cucumber through priming application has been made. Our goal was also to examine the expression levels of selected ncRNAs at an earlier of response to hypoxia stress (2 days). Interaction between differentially expressed lncRNAs and miRNAs was examined to determine potential regulatory pathways.

2. Results

2.1. LncRNAs Identified in Cucumber under Long-Term Waterlogging

The total number of identified lncRNAs was 3738, which accounted for 10% of all identified transcripts (35712) in de novo assembled transcriptomes for unstressed (Ctrl), non-primed, once waterlogged plants (1xH) and primed, twice waterlogged plants (2xH) [7] (Supplementary Materials Data S1). The largest percentage of lncRNAs (37%) was classified as exonic overlap with reference on the opposite strand ('x') and 19% were classified as unknown intergenic transcripts ('u') (Figure 1a). Identified lncRNAs were distributed across all cucumber chromosomes, and the highest number of lncRNAs was located on chromosome 3, and later on chromosome 6 (Figure 1b). Approximately 15% of identified lncRNAs were longer than 2000 bp (Figure 1c).

(a)

Cufflink class		Number	Percentage
Description	Code		
Exonic overlap with reference on the opposite strand	x	1400	37%
Intergenic lncRNA	u	703	19%
Complete match of intron chain	=	648	17%
Potentially novel isoform (fragment): at least one splice junction is shared with a reference intron	j	666	18%
Generic exonic overlap with a reference transcript	o	308	8%
Intronic lncRNA	i	13	1%
Total		**3738**	

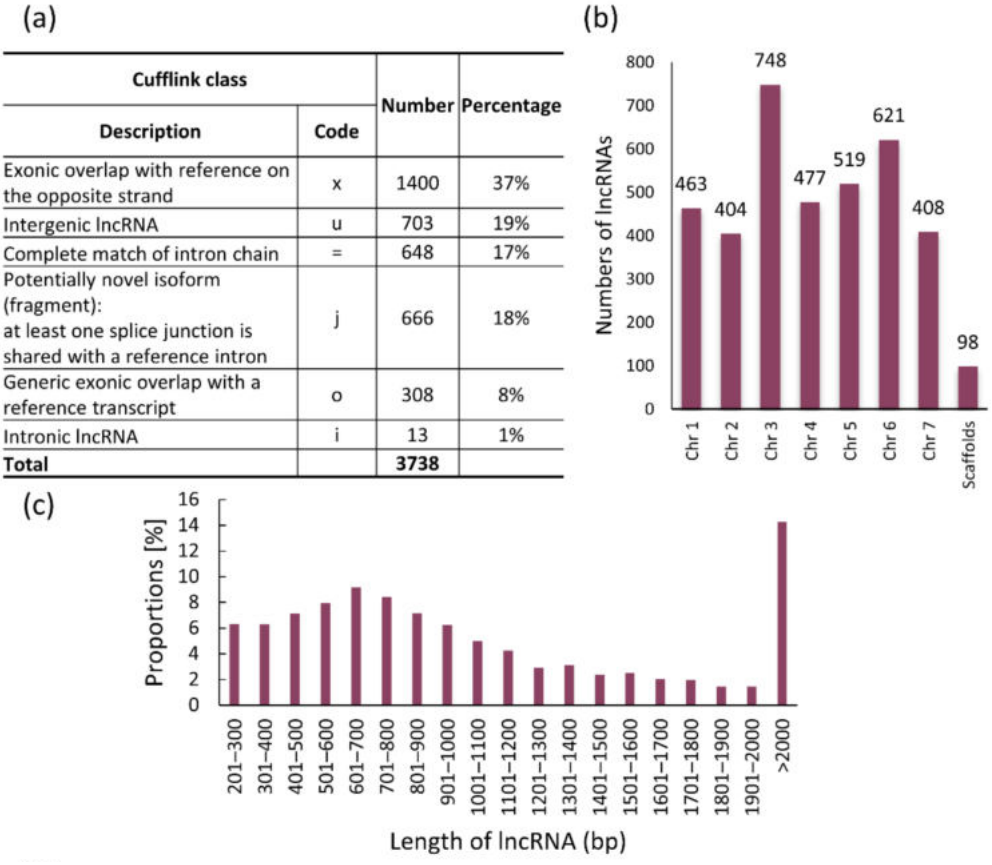


Figure 1. *Cont.*

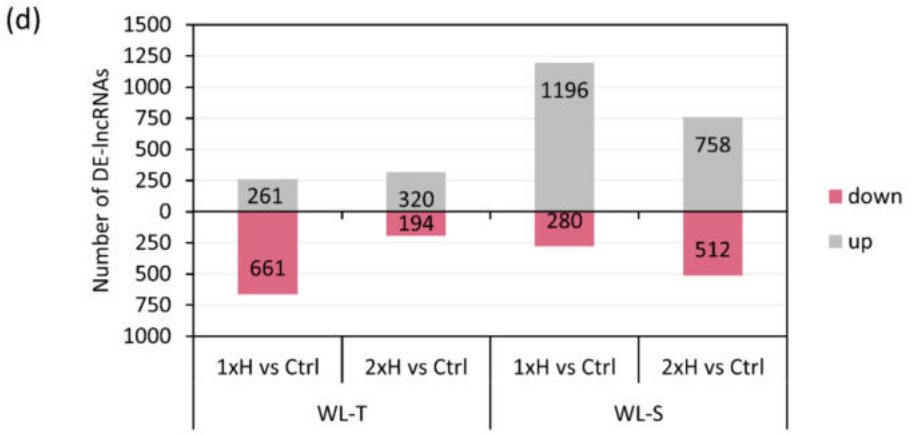


Figure 1. Characterization of lncRNAs in two cucumber accessions (WL-T and WL-S) under long-term waterlogging. (**a**) Classification of lncRNAs on the basis of their genomic locations with respect to adjacent protein coding genes. (**b**) Distribution of lncRNAs across chromosomes. (**c**) Transcript size distributions for all lncRNAs. (**d**) Number of DE-lncRNA (Differentially Expressed lncRNA) identified in each performed comparison with control conditions in both cucumber accessions. WL-T—waterlogging tolerant accession, WL-S—waterlogging sensitive accession, Ctrl—unstressed plants, 1xH—non-primed, once waterlogged plants, 2xH—primed, twice waterlogged plants.

Analysis of differential expression showed the highest number of DE-lncRNAs (Differentially Expressed lncRNAs) in comparison to control conditions in waterlogging sensitive (WL-S) accession (DH4) after induction of 7 days of waterlogging (1xH), i.e., 1476, of which 81% were up-regulated. In primed plants of WL-S (2xH), the total number of DE-lnRNAs was 1270, while the smallest number of regulated genes were detected in the waterlogging-tolerant (WL-T) accession (DH2) under the second waterlogging treatment (2xH), i.e., 514 (62% of them upregulated) (Figure 1d, Supplementary Materials Data S2–S5).

We also determined the number of DE-lncRNAs according to the genome location classification in each comparison, separating up-regulation and down-regulation of those lncRNAs (Figure 2). A total of 60% of DE-lncRNAs with enhanced expression in WL-S after 7 days of hypoxia were classified to exonic overlap with reference on the opposite strand (x), whereas in WL-T accessions, only 18% (47) lncRNAs were assigned to that class (Figure 2). This may indicate a potential mechanism of gene regulation under the long-term waterlogging.

In total, 922 and 1476 lncRNAs were differentially regulated in WL-T and WL-S, respectively, with 743 molecules shared in both accessions after 7 days of long-term waterlogging (non-primed, 1xH) (Figure 3a), and 482 after second exposure to stress (primed, 2xH) (Figure 3b). A total of 303 differentially expressed lncRNAs (DE-lncRNA) were identified across all treatment groups compared to control samples (Figure 4). The highest number of specific DE-lncRNAs was found in WL-S after a single hypoxia treatment (1xH), 299, whereas only 9 unique molecules were assigned to WL-T after second hypoxia treatment (2xH). We also indicated 71 DE-lncRNAs potentially involved in acquiring tolerance to oxygen deprivation, since they were regulated in non-primed in WL-T accession and in primed plants of WL-S (Supplementary Materials Data S6). Among those lncRNAs, the expression level of seven was enhanced, whereas inhibition was displayed by 61 of them in both accessions. TCONS_00009645 and TCONS_00019419 had different expression pattern between accessions, i.e., in WL-T they were up-regulated, while in WL-S they were down-regulated.

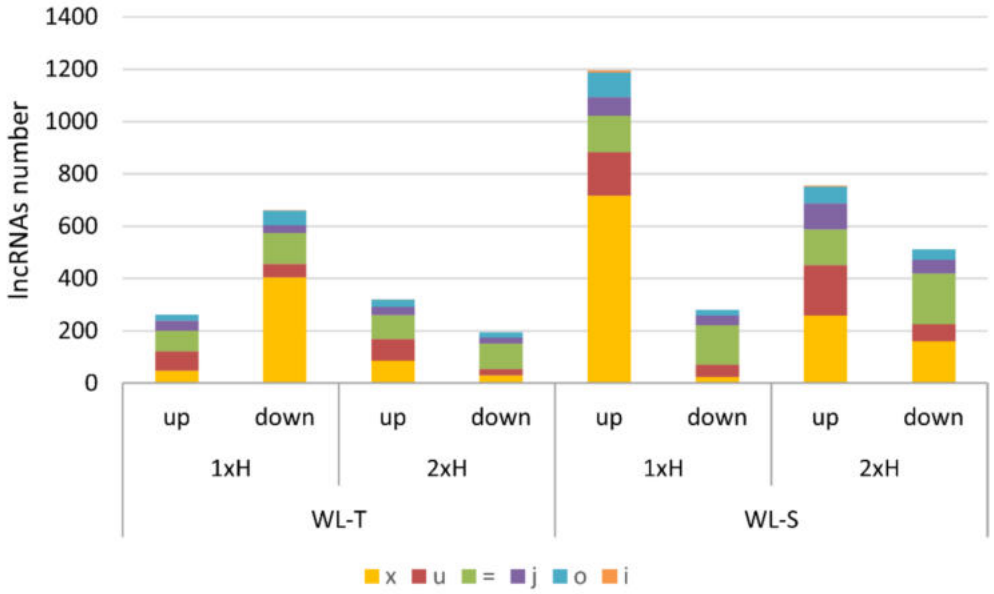


Figure 2. Distribution of lncRNAs due to genome localization among differentially expressed lncRNAs in two cucumber accessions (WL-T and WL-S) under waterlogging (1xH and 2xH) in comparisons to control conditions. 'x'—exonic overlap with reference on the opposite strand, 'u'—intergenic lncRNA, '='—complete match of intron chain, 'j'—potentially novel isoform (fragment): at least one splice junction is shared with a reference intron, 'o'—generic exonic overlap with a reference transcript, 'i'—intronic lncRNA.

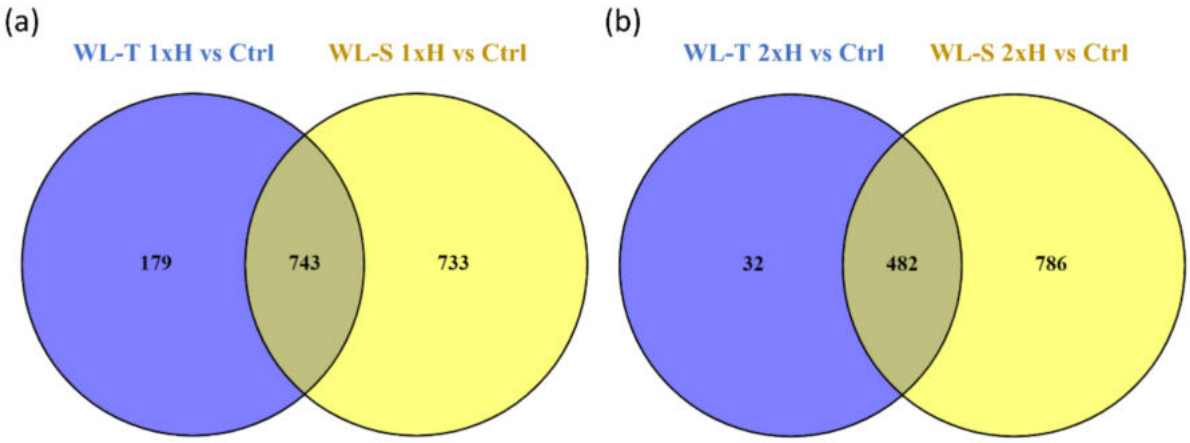


Figure 3. Venn diagrams showing the number of differentially expressed lncRNAs that are commonly and uniquely regulated in: (**a**) non-primed (1xH) and (**b**) primed (2xH) plants in two cucumber accessions, WL-T and WL-S.

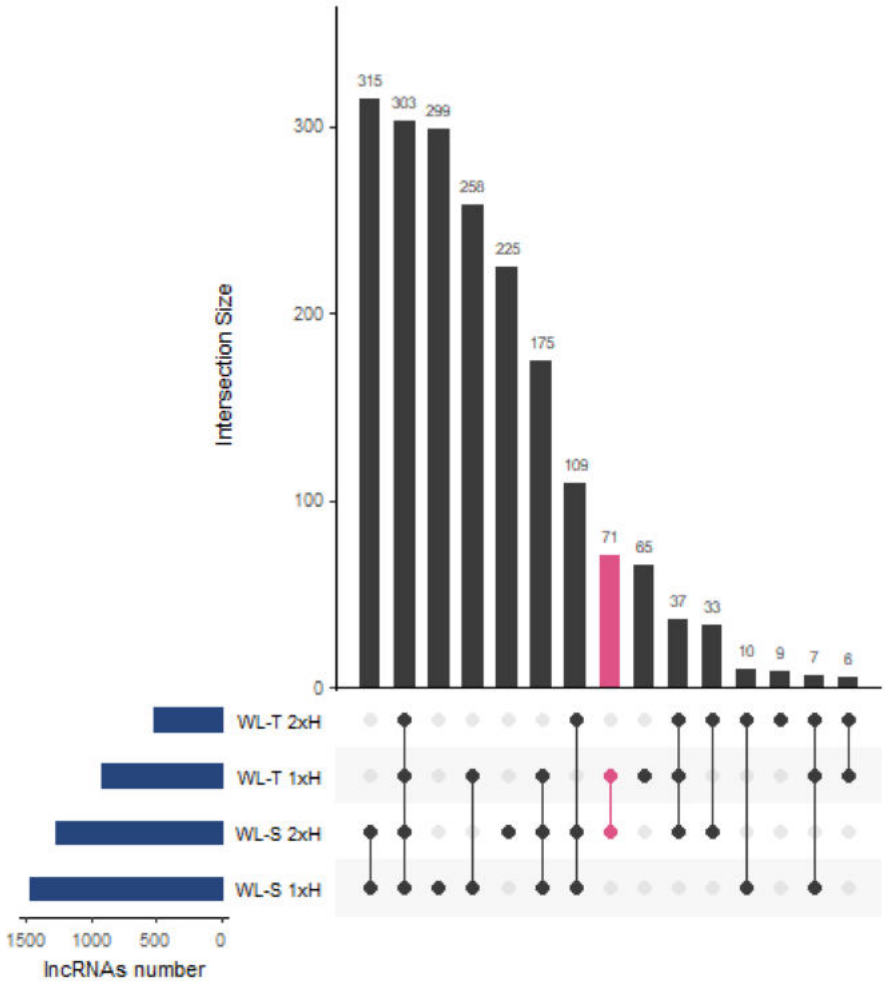


Figure 4. Upset plot presenting number of common and specific DE-lncRNA in four treatment groups in comparison to control conditions, i.e., non-primed (1xH) and primed (2xH) plants in two cucumber accessions, WL-T and WL-S. In pink, number of lncRNAs potentially involved in acquisition of long-term tolerance to waterlogging was shown.

2.2. Target Genes for DE-LncRNAs

The genes located in the nearest location (down- and upstream) to the identified lncRNAs were considered as potential target genes. It was found that 3036 lncRNAs could potentially regulate expression of 2209 proximal genes (Supplementary Materials Data S1). In non-primed plants (1xH), 797 and 407 genes were identified as potentially regulated by DE-lncRNA in WL-T and WL-S accessions, respectively. In the case of primed plants (2xH), 402 and 1100 of genes were indicated as lncRNAs targets for WL-T and WL-S.

GO enrichment analysis was conducted to determine the biological process and molecular functions (MF) in which potential targets genes are involved (Figure 5). The common significant GO terms for Biological Process (BP) in both accessions were response to wounding (GO:0009611) and negative regulation of endopeptidase activity (GO:0010951), whereas serine-type endopeptidase inhibitor activity (GO:0004867) was found to commonly enrich the Molecular Function (MF) category in response to oxygen deprivation in both accessions. DNA-binding transcription factor activity (GO:0003700) was an enriched term in non-primed WL-T and primed WL-S plants, highlighting its link to hypoxia stress in cucumber.

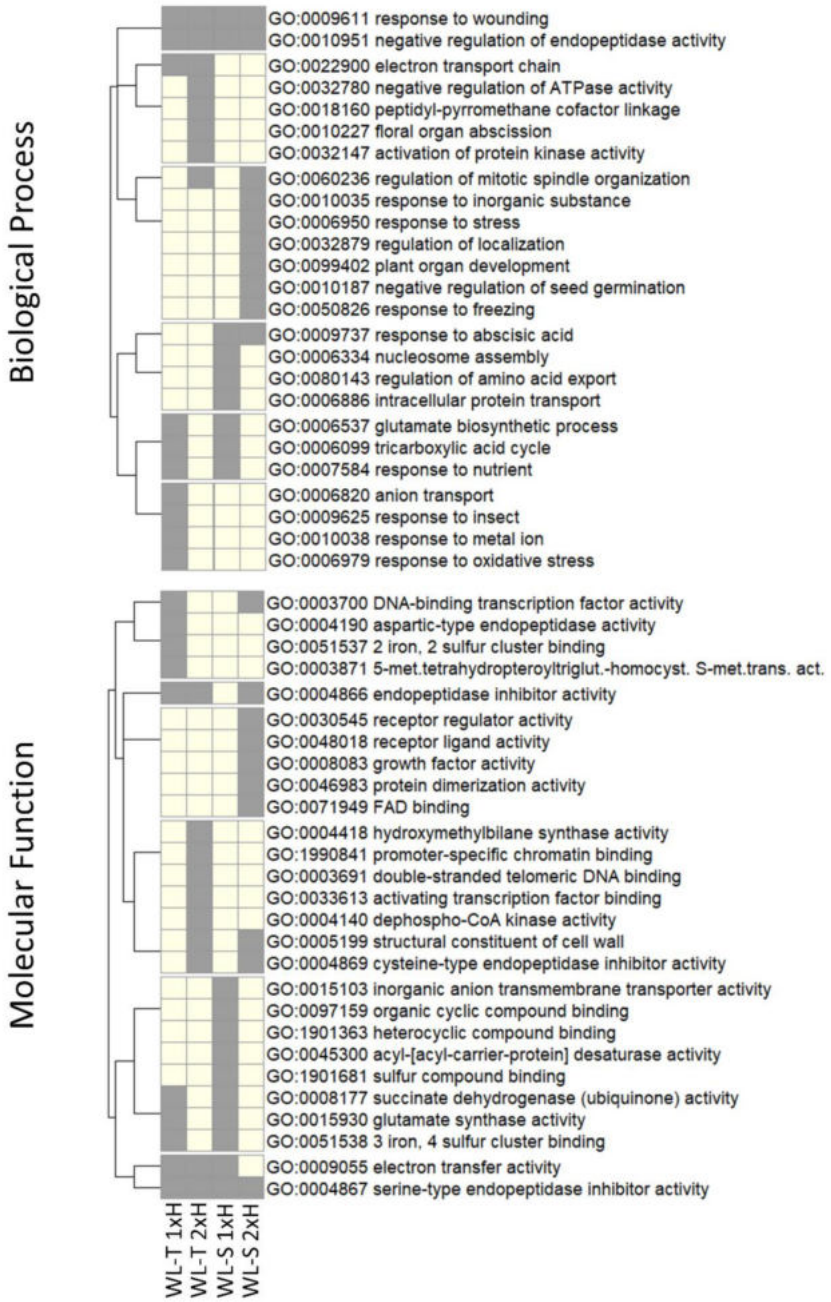


Figure 5. The top enriched GO categories in the Biological Process (BP) and Molecular Function (MF) determined for DE-lncRNAs target genes in non-primed (1xH) and primed (2xH) plants in two cucumber accessions, i.e., WL-T and WL-S under long-term waterlogging. Grey colour means presence of the term, whereas white colour indicates lack of statistically significant enrichment for the term.

2.3. Validation of lncRNAs with Quantitative Real-Time PCR (QRT-PCR)

From the identified lncRNA molecules, eight were selected and validated with qRT-PCR (Table 1). The selected molecules revealed in the RNAseq data [7], among others, the opposite regulation in both cucumber accessions in non-primed plants. Additionally, they demonstrated different expression levels between non-primed (1xH) and primed (2xH) in both accessions, which may indicate that these molecules potentially play a role in waterlogging stress tolerance.

Table 1. List of lncRNA molecules selected for validation using qRT-PCR on the basis of the RNAseq data [7].

lncRNA	WL-T		WL-S		Class Code	Nearest Gene	Gene Description	Gene Ontology
	1xH *	2xH **	1xH	2xH				
TCONS_00003967	−4.57	ns ***	6.93	ns	x	Csa1M422990.1	Xyloglucan endotransgluco-sylase/hydrolase	-
TCONS_00008071	−4.66	ns	7.44	ns	x	Csa2M174150.1	Malate dehydrogenase	BP: GO:0006099
TCONS_00015763	−8.06	ns	6.62	−4.41	x	Csa3M782680.1	Syntaxin, putative	BP: GO:0009737
TCONS_00019433	−7.42	ns	7.13	ns	x	Csa4M054300.1	26S proteasome non-ATPase regulatory subunit	-
TCONS_00014209	ns	ns	5.51	ns	u	-	-	-
TCONS_00019494	ns	ns	8.87	ns	x	Csa4M063450.1	ATP-dependent RNA helicase, putative	MF: GO:0097159, GO:1901363
TCONS_00032986	ns	ns	6.07	ns	x	Csa7M357030.1	Transcription initiation factor TFIID subunit 1-A	-
TCONS_00021873	5.23	7.34	ns	3.50	u	-	-	-

* Differences in expression between non-primed (1xH) and unstressed plants (Ctrl), ** differences in expression between primed (2xH) and unstressed plants (Ctrl), *** ns—no statistically significant differences in expression.

Expression of all selected lncRNAs differentiates the studied cucumber accessions (Figures 6 and 7). For example, after first waterlogging treatment (1xH) in WL-S, TCONS_00003967, TCONS_00008071, TCONS_00015763 and TCONS_00019433 were up-regulated, while in the WL-T accession, expression levels of these lncRNAs were reduced in comparison to control conditions (Figure 6). In the cases of TCONS_00014209, TCONS_00019494 and TCONS_00032986, their expression was enhanced in non-primed plants of WL-S (after 7 days of first hypoxia treatment), whereas in the WL-T accession, expression remained unchanged in comparison to control plants (Figure 7a–c). Only one lncRNA (TCONS_00021873) was overexpressed after 7 days of stress in WL-T, whereas in WL-S, no regulation was detected (Figure 7d).

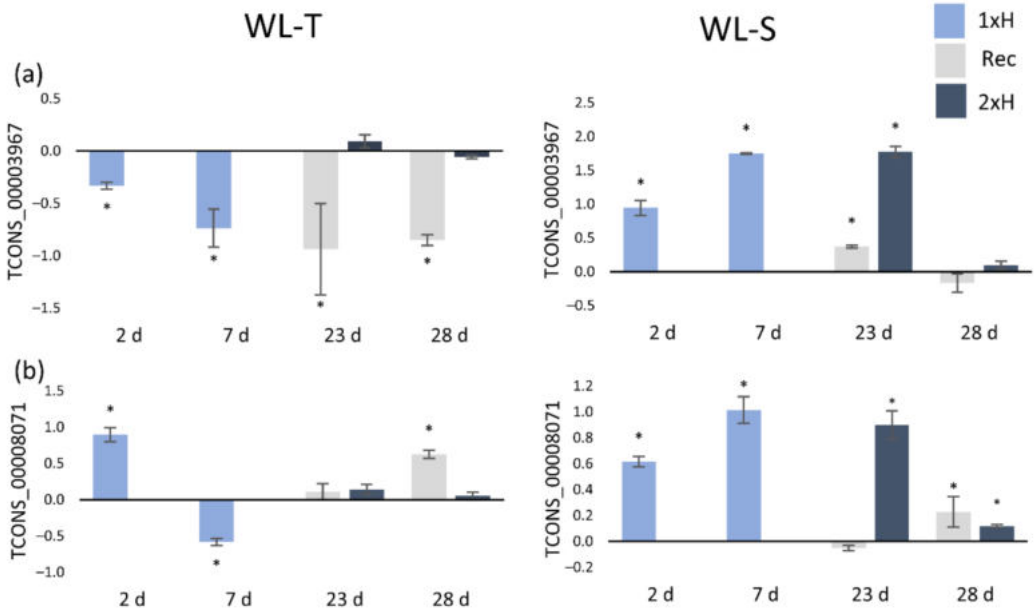


Figure 6. *Cont.*

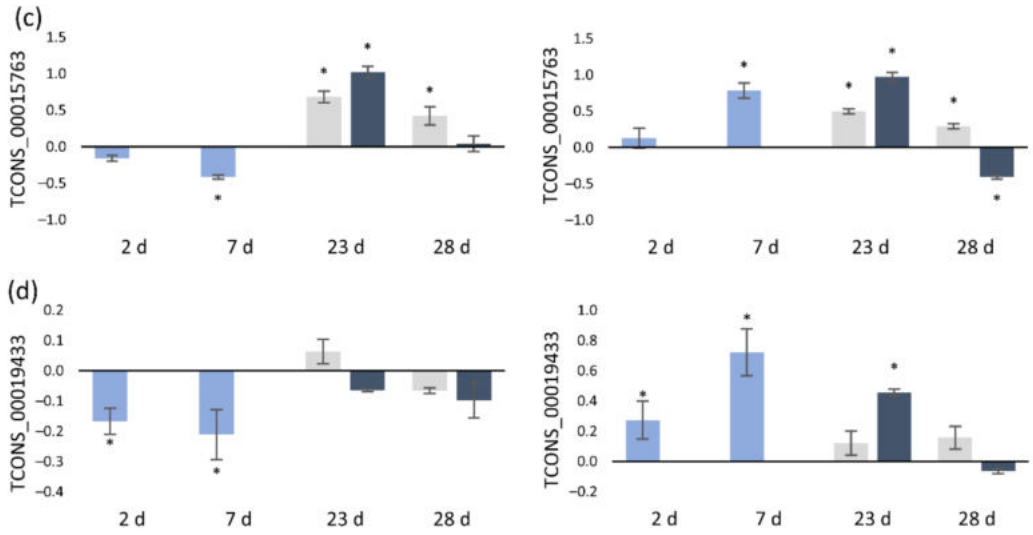
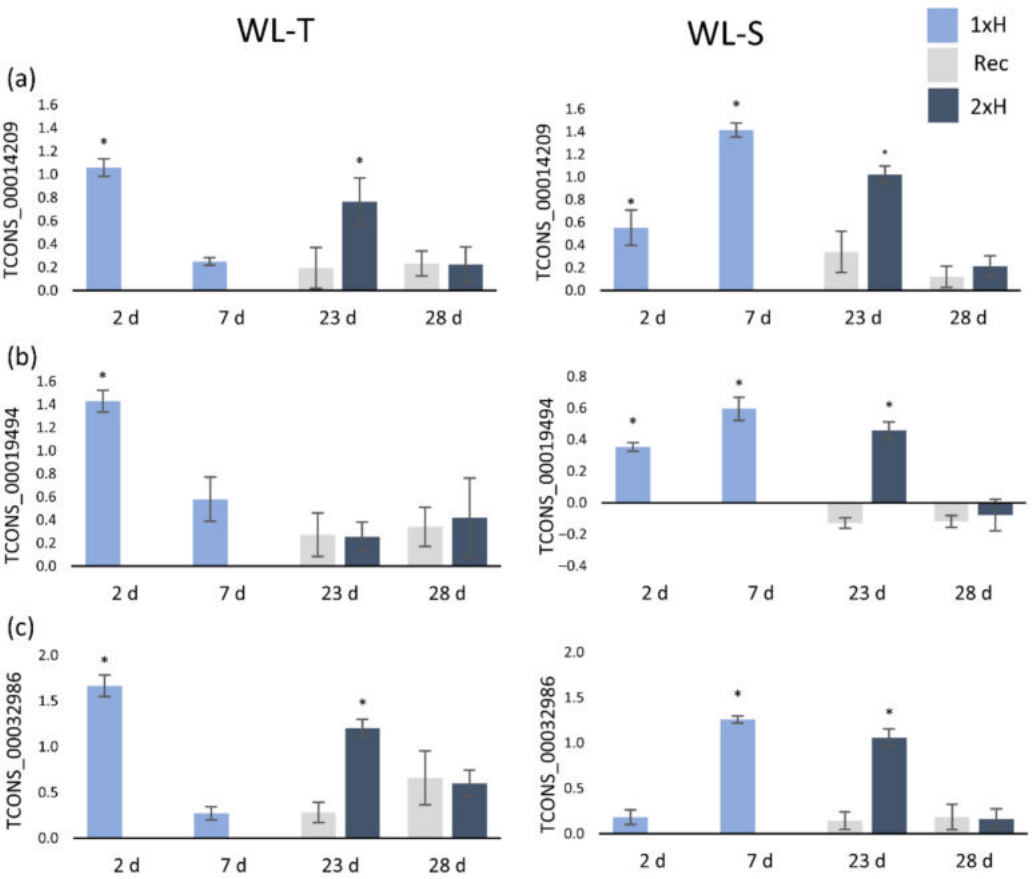


Figure 6. Expression profiles of four lncRNAs, i.e., TCONS_00003968 (**a**), TCONS_00008071 (**b**), TCONS_00015763 (**c**), and TCONS_00019433 (**d**), depicting differences in the expression levels in non-primed plants in WL-T and WL-S cucumber accessions under waterlogging stress. 1xH—non-primed plants, Rec—non-primed plants after 14-day recovery period, 2xH—primed plants. Data are expressed as the mean ± SD (standard deviation) of three independent biological replicates and three technical replications. Asterisks indicate a significant difference vs. control plants.



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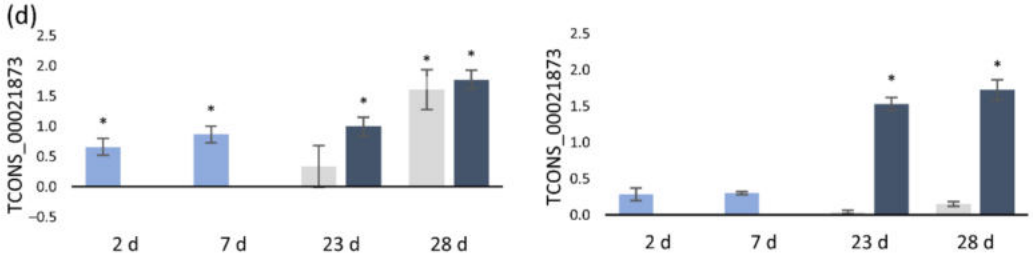

Figure 7. Expression profiles of four lncRNAs, i.e., TCONS_00014209 (**a**), TCONS_00019494 (**b**), TCONS_00032986 (**c**), and TCONS_00021873 (**d**) in WL-T and WL-S cucumber accessions under waterlogging stress. 1xH—non-primed plants, Rec—non-primed plants after 14-day recovery period, 2xH—primed plants. Data are expressed as the mean ± SD (standard deviation) of three independent biological replicates and three technical replications. Asterisks indicate a significant difference vs. control plants determined with Student *t*-test, $p < 0.05$.

Exposure of plants to another waterlogging treatment (2xH) led to up-regulation of these lncRNAs after 2 days in WL-S, while in WL-T, this could be observed for only some of them (TCONS_00015763, TCONS_00014209, TCONS_00032986 and TCONS_00021873) (Figures 6c and 7a,c,d). In both primed WL-T and WL-S plants, i.e., after the second treatment, only the expression level of TCONS_00021873 was enhanced (Figure 7d). In WL-S, upregulation after 2 days of stress, and repression after 7 days were detected for all lncRNAs except TCONS_00021873 (Figures 6 and 7a–c).

Validation by qRT-PCR indicated a specific overexpression of TCONS_00032986 and TCONS_00021873 after 2 days of stress induction only in WL-T (Figure 7c,d), which was not observed in WL-S accession in early response to stress. TCONS_00021873 was consistently expressed in WL-T at each time-point, whereas in WL-S, upregulation was detected only in primed plants (2xH) (Figure 7d).

2.4. miRNAs Involved in Response to Long-Term Waterlogging Stress

In total, 489,977,941 raw reads were obtained from 18 libraries, of which 252,796,468 reads were from WL-T accession and 237,181,473 reads were from WL-S accession libraries (Supplementary Table S1). As expected, the highest fraction of reads was 21–24 nucleotides long (Figure 8) in both accessions. Most reads were obtained from a library derived from WL-T control plants, and the length of these sequences was 24 bp (Figure 8). In WL-S, the number of reads of each length was similar between the libraries representing control (Ctrl) and primed plants (2xH). This relation was not observed for the number of reads in the DH2 accession. In total 684 miRNAs were detected in all samples (data available on request from the authors).

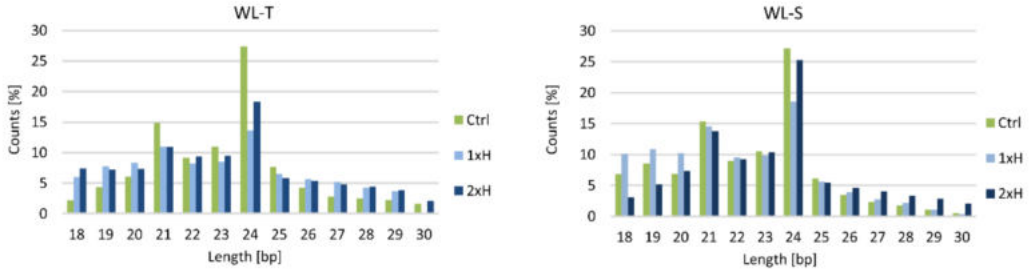

Figure 8. The length distribution for small RNA sequences WL-T and WL-S cucumber accessions of Ctrl, 1xH and 2xH experimental groups.

2.5. Long-Term Waterlogging-Responsive Novel miRNAs in Cucumber

Identification of differently expressed miRNAs (DE-miRNAs) under oxygen deprivation was performed with edgeR programme. This led to the discovery of only 19 miRNAs presenting differential expression between non-primed (1xH), primed (2xH) and control

(Ctrl) plants of both cucumber accessions, respectively. For 5 of 19 DE-miRNAs analysis with the use of ShortStack software confirmed these miRNAs to be novel (Supplementary Materials Data S7).

The highest number of DE-miRNAs, 10, was found in primed plants of WL-T, whereas only five DE-miRNAs were depicted in non-primed plants of the WL-S accession (Figure 9). Of the 19 long-term waterlogging-responsive miRNAs, csa-novel_miR1 was strongly up-regulated in non-primed and primed plants of both accessions in response to long-term waterlogging (Figure 10). Three of the identified miRNAs, i.e., csa-novel-miR4/miR169, csa-novel_miR8 and csa-novel_miR18 molecules, were determined as being uniquely up-regulated in non-primed and primed plants of WL-T and primed plants of WL-S, respectively. Differences in expression levels of miRNAs between the treatments and accessions were observed, which may indicate a different response/tolerance to oxygen restricted stress.

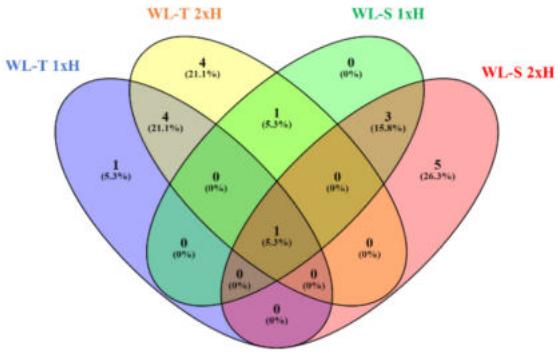

Figure 9. Venn diagram with common and specific miRNAs differentially expressed under hypoxic conditions (1xH, 2xH) in two cucumber accessions, i.e., WL-T and WL-S, compared to control.

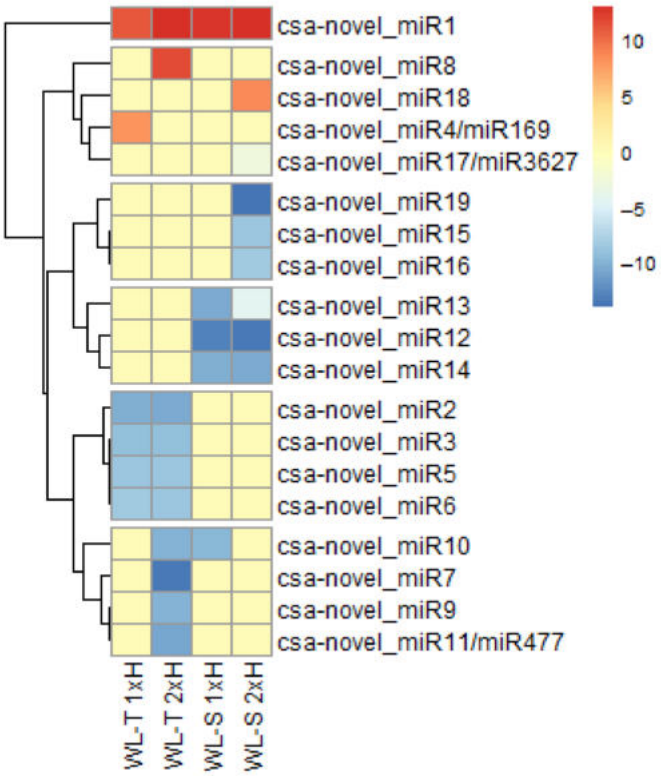

Figure 10. miRNAs differentially expressed in non-primed (1xH) and primed (2xH) cucumber WL-T and WL-S accessions under long-term waterlogging. The heatmap represents $\log_2$FC values in comparison to control conditions with FDR < 0.05.

The highest number of specific miRNAs was identified in primed plants (2xH) of the WL-S accession, while in non-primed plants, no specific miRNAs with differential expression were identified (Figure 9). For WL-T, one and four uniquely regulated miRNAs were found under long-term waterlogging in non-primed and primed plants, respectively.

The most up-regulated specific miRNA was csa-novel_miR8 ($log_2FC = 11.44$) and regulation was observed in WL-T accession in primed plants (2xH). In turn, in primed plants of the WL-S accession, csa-novel_miR19 was mostly inhibited under oxygen deprivation (Figure 10).

2.6. QRT-PCR of miRNAs Involved in Long-Term Waterlogging

For qPCR assay, the miRNA with the highest expression level in response to waterlogging stress in both cucumber accessions was chosen (csa-novel_miR1). Additionally, we randomly selected miRNAs with stable expression between non-primed, primed and control plants, but with a high number of normalized reads in order to examine the early stage of response (csa-novel_miR20, csa-novel_miR21). To confirm hypoxic conditions, we chose csa-miR-394a [55].

The enhanced expression of csa-novel_miR1 was detected in both accessions after 2 and 7 days in non-primed plants, while second treatment caused up-regulation only after 7 days of waterlogging (2xH) (Figure 11a). The expression of csa-novel_miR20 was enhanced after 2 days of first waterlogging treatment in both accessions; however, during the second treatment, expression of that miRNA was enhanced only in WL-S at the 2 day time-point (Figure 11b). Waterlogging treatment had an influence on differential expression of csa-novel_miR21 only in WL-S, with its up-regulation being observed after 2 days of first (1xH) and second stress (2xH) induction. In WL-T, differences between control and non-primed and primed, as well, were not detected (Figure 11c). Analysis of the expression pattern of csa-mir394a revealed increased expression levels on the second day of both waterlogging treatments in WL-T, whereas in WL-S, expression was inhibited after two days of the second treatment (Figure 11d). Long-term waterlogging did not have any impact on differential expression of csa-miR394a in cucumber.

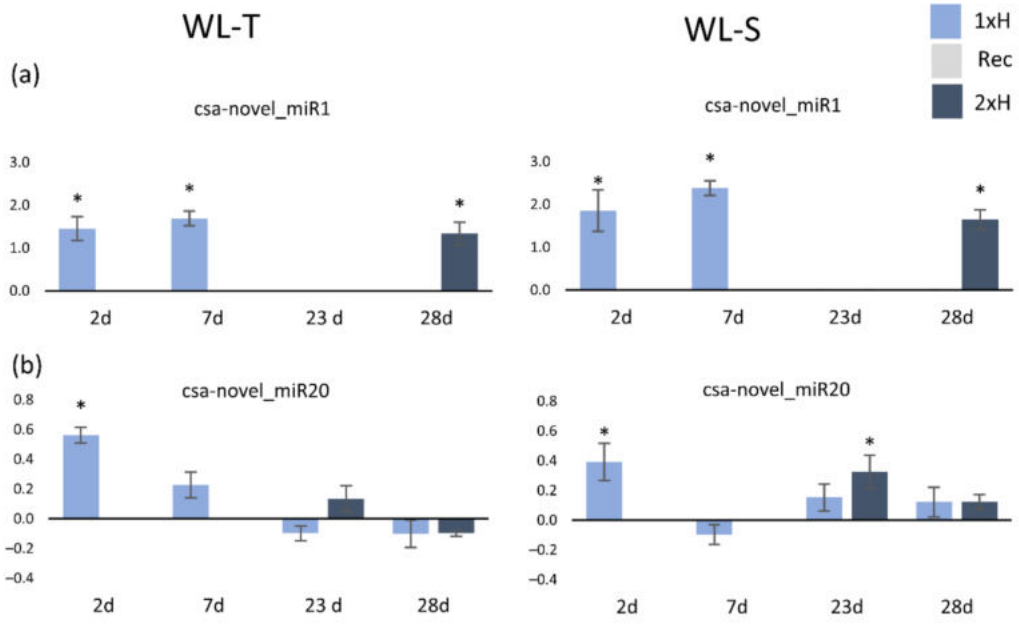

Figure 11. *Cont.*

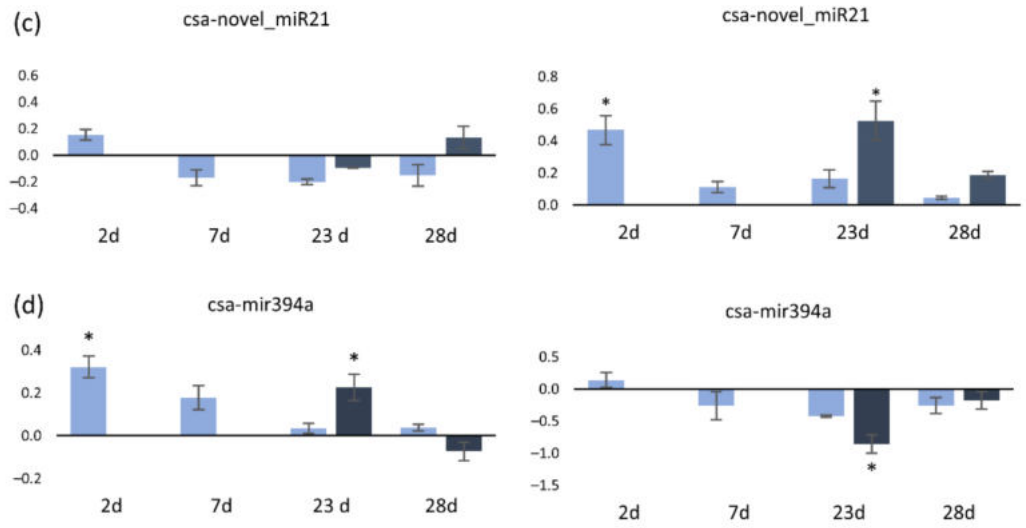

Figure 11. Expression level of three novel miRNAs, i.e., csa-novel_miR1 (**a**), csa-novel_miR20 (**b**), csa-novel_miR21 (**c**) and one known csa-miR394a (**d**) determined in non-primed and primed plants of WL-T and WL-S cucumber accessions. 1xH—non-primed plants, Rec—non-primed plants after 14-day recovery period, 2xH—primed plants. Data are the mean of three independent replicates. Asterisks indicate a significant difference vs. control plants determined with Student t-test, $p < 0.05$.

2.7. Interaction between LncRNAs and miRNAs

A total of 208 DE-lncRNAs were revealed as potential targets for 207 miRNAs in cucumber under long-term waterlogging (Supplementary Materials Data S8). From the pool of all interactions, we selected the 71 lncRNAs that were possibly involved in acquiring tolerance to long waterlogging in cucumber to examine the potential role of miRNAs in their expression regulation. Six of the 71 lncRNAs were possibly targeted by seven miRNAs (Supplementary Table S2). TCONS_00004681 may be a target of three miRNAs. Five target lncRNAs were down-regulated in non-primed plants of WL-T and primed plants of WL-S, except TCONS_00030467, potentially depicted as one of two targets for csa-novel_miR440, as its expression was enhanced (Figure 12).

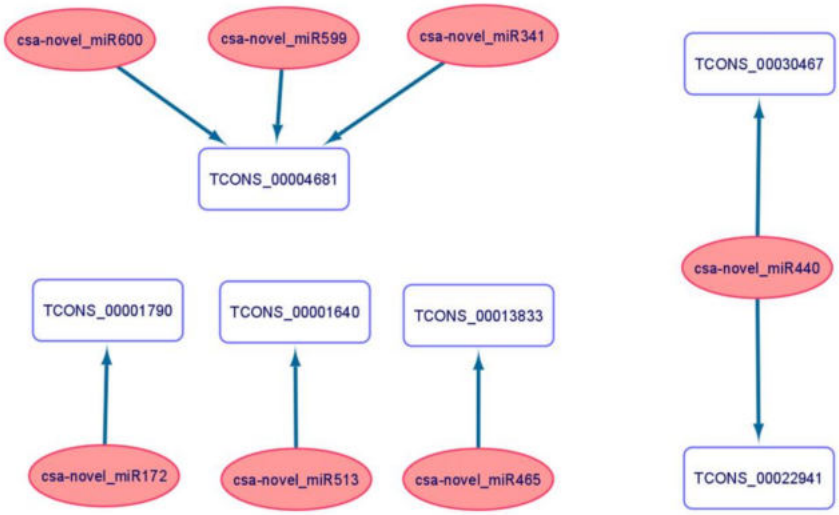

Figure 12. Interaction network analysis representing miRNAs (pink circles) with target lncRNAs (blue round rectangle).

Moreover, we found that five lncRNAs could act as endogenous target mimics (eTMs) for three DE-miRNAs. Interestingly, those miRNAs were down-regulated under long-term waterlogging (Figure 13).

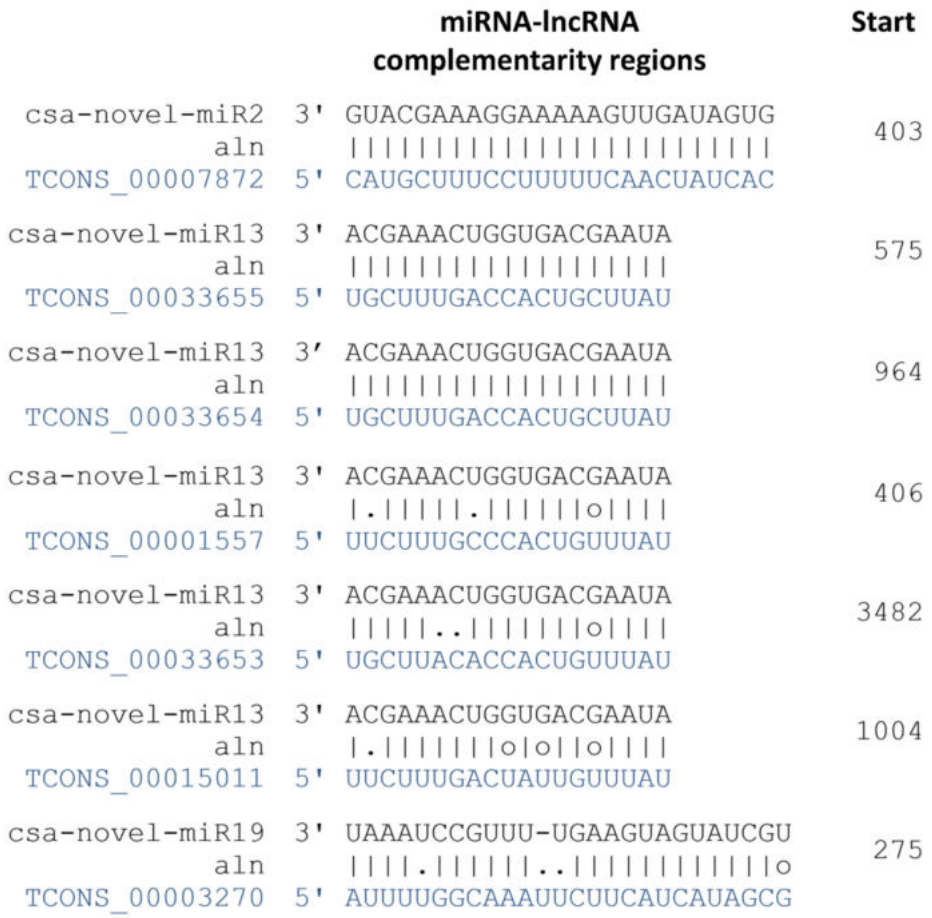

Figure 13. Identified endogenous target mimics (eTMs) of miRNAs. The '.', '−', 'o', and '|' represent mismatches, gaps, G:U pairs, and complementary bases, respectively.

3. Discussion

Genes participating in the response to stresses can be regulated at transcriptional and post-transcriptional levels, among others, by ncRNAs. High-throughput sequencing assays make it possible, with better specificity, to detect those ncRNAs [32]. Understanding of the mechanisms participating in gene regulation under long-term waterlogging in cucumber will provide essential knowledge, making it possible to investigate accessions with enhanced tolerance to oxygen deprivation.

3.1. LncRNAs Differentially Expressed under Long-Term Waterlogging

About 37% of all identified lncRNAs in cucumber under long-term waterlogging treatment were classified in terms of their location in the genome into a group of molecules that exonically overlap with a target gene on the opposite strand, which may indicate their role in the silencing of gene expression. The highest number of overexpressed lncRNAs classified to this class was identified in WL-S in non-primed plants.

Differences in the number of regulated lncRNAs between tolerant and sensitive genotypes were also determined in *Brassica napus* L. under drought stress and re-watering [56]. A higher number of DE-lncRNAs was indicated in the sensitive genotype, similar to results obtained in our research. These results confirm differences between cucumber accessions in response to long-term waterlogging. The number of DE-lncRNAs in primed plants of WL-S accession decreased in comparison to the non-primed plants, meaning fewer genes was regulated. This may suggest that another exposure to stress is less harmful for the cucumber plants.

LncRNAs can regulate expression of genes located nearby [57]. In our study, 2209 genes were predicted as being potentially targeted by 3036 lncRNAs. GO enrichment anal-

ysis was performed for target genes of DE-lncRNAs in non-primed and primed cucumber accessions under long-term waterlogging. We observed a diversity of GO terms enriched by target genes in each comparison, confirming differences in response to hypoxia in both cucumber accessions. In non-primed plants of WL-T accession, target genes were specifically enriched in processes involved in response to oxidative stress (GO:0006979) caused by reactive oxygen species (ROS) production. Overrepresentation of ROS can lead to damage of cellular components, such as membrane lipids, and as a consequence to cell death, so it is important to activate the mechanism to prevent this [58]. Balance between ROS production and its scavenging is related to waterlogging-tolerant species [59]. Regulation of genes involved in the response to oxidative stress under oxygen deprivation was also observed in tolerant cucumber accession [60].

DE-lncRNAs target genes in non-primed plants of the WL-S accession were specifically enriched with respect to nucleosome assembly (GO:0006334), regulation of amino acid export (GO:0080143), and intracellular protein transport (GO: 0006886). Exposure of the plants to unfavourable environmental conditions must immediately be responded to by changes at the biochemical and physiological levels caused by the regulation of gene expression, whereby a fundamental role is played by nucleosome assembly. Highly conserved histone chaperones are involved in nucleosome assembly, leading to remodelling of the chromatin structure during transcription or DNA replication [61,62]. Enrichment in the nucleosome assembly category was noted only in the non-primed plants of WL-S, which may indicate that chromatin organization is more advanced upon oxygen deprivation in WL-S than in WL-T. Functional prediction showed that target genes potentially regulated by lncRNAs in non-primed plants of WL-S were also specifically enriched in processes connected with transport inside the cells and between the plant organs in order to maintain homeostasis in the cells and to transport, among other things, a reduced form of nitrogen [63,64].

qRT-PCR validation of selected lncRNAs was consistent with results obtained using the RNA-Seq approach. We depicted four lncRNAs, i.e., TCONS_00003967, TCONS_00019433, TCONS_00032986, and TCONS_00021873, that can be used for cucumber differentiation regarding their tolerance to hypoxia stress on just the second day of long-term waterlogging. Differences in lncRNA expression levels determined by qRT-PCR between accessions have also been established in Chinese cabbage under heat stress conditions [65]. In our study, TCONS_00003967 and TCONS_00019433 were up-regulated in WL-S, whereas TCONS_00032986 and TCONS_00021873 presented over-expression in WL-T. In the context of priming and acquiring tolerance to hypoxia, we observed that TCONS_00021873 was up-regulated in WL-T, whereas in WL-S it was not differentially expressed, but its expression was enhanced in primed plants, meaning that priming resulted in regulation of that lncRNA molecule. TCONS_00021873 was classified as an intergenic lncRNA, so its function needs to be further elucidated, for example by genome editing.

Six among eight validated lncRNAs were classified according to the location in the genome as exonic overlapped with a reference on the opposite strand, which potentially means that expression of co-located target-genes may be regulated by those lncRNAs. We showed that TCONS_00003967 could potentially regulate the xyloglucan endotransglucosylase/hydrolase gene, TCONS_00008071 can target the malate dehydrogenase gene, TCONS_00015763—the syntaxin gene, TCONS_00019433 can influence the gene-encoded 26S proteasome non-ATPase regulatory subunit, TCONS_00019494 can be located close to the ATP-dependent RNA helicase gene, and finally, TCONS_00032986 can regulate the transcription initiation factor TFIID subunit 1-A gene.

Our results revealed a down-regulation of lncRNA (TCONS_00003967), potentially targeting the xyloglucan endotransglucosylase/hydrolase gene, in non-primed WL-T and up-regulation of this lncRNA in WL-S, and additionally, we found a down-regulation of xyloglucan endotransglucosylase/hydrolase in the WL-S accession in the RNA-Seq assay, suggesting that TCONS_00003967 inhibits expression of the target genes due to its antisense location. Overexpression of gene-encoded xyloglucan endotransglucosylase/hydrolase re-

sults in enhanced flooding tolerance in soybean [66] by modifying the cell wall architecture, leading to adaptation to waterlogging [67].

lncRNA, i.e., TCONS_00008071, which potentially regulates the malate dehydrogenase gene was down-regulated in the WL-T and up-regulated in the WL-T accession in non-primed plants. Compared to RNA-Seq data, the malate dehydrogenase gene presented diverse expression patterns, which may indicate that the lncRNA TCONS_00008071 regulates target gene expression at the transcriptional level by inhibition. In research described by Qi et al. [47] on the effects of hypoxia on gene expression level in tolerant accessions of cucumber, malate dehydrogenase was down-regulated; however, data in that research were established at 24 h after start of waterlogging, and it can be said that the expression level of malate dehydrogenase changes with the duration of oxygen deprivation, and is enhanced with long-term waterlogging treatment, which was also detected in barley after 3 weeks of stress [68]. Malate dehydrogenase is also involved in tolerance to abiotic stresses, such as cold and salt by, among others, reducing the levels of superoxide anion (0^{2-}) [69].

In our studies, we also determined the syntaxin gene to be a potential target, up-regulation of which led to tolerance to oxidative stress [70]. We observed up-regulation of TCONS_00015763 in non-primed plants of the WL-S accession and down-regulation of this lncRNA in non-primed plants of the WL-T and primed plants of the WL-S accession, resulting in the expression level of the target gene in this group, i.e., syntaxin, being detected as overexpression in non-primed WL-T plants and primed WL-S plants. This suggests that primed WL-S plants may acquire tolerance to oxidative stress, causing, among other things, damage to carbohydrates, amino-acids and lipid membranes during hypoxia stress in plants [58]. In its interaction with lncRNA-target genes, TCONS_00015763 may regulate the expression of target genes through inhibition of expression. Further study is required regarding TCONS_00015763 as a syntaxin regulator in acquiring tolerance to hypoxia stress.

The same expression pattern in terms of lncRNA–target gene interaction as that found in the previously described genes was also observed for TCONS_00019433, which can potentially regulate the 26S proteasome non-ATPase regulatory subunit (*RPN1A*) gene. RPN1A is needed for optimal growth and response to stress (https://www.uniprot.org/uniprot/Q9SIV2, accessed on 28 May 2021). This lncRNA–target gene interaction may be another example of the influence of priming on enhancing tolerance to long-term waterlogging. In the literature, there is lack of strict evidence of the participation of the 26S proteasome non-ATPase regulatory subunit in the response to waterlogging in cucumber, so this is the first information found regarding the potential role of TCONS_00019433–26S proteasome non-ATPase regulatory subunit in developing tolerance to hypoxia in cucumber.

It was found that TCONS_00019494 can influence the expression of the ATP-dependent RNA helicase gene. Overexpression of that lncRNA was only observed in non-primed plants of the WL-S accession, and simultaneously, expression of target-genes was also enhanced. This suggests that TCONS_00019494 does not affect the target gene through inhibition of expression. This mechanism needs to be further elucidated.

Deeper analysis and exploration are needed to understand interactions of lncRNAs and potential target genes, especially those located close to protein-coding genes, in terms of their expression and influence on tolerance to hypoxia stress in cucumber. Understanding of the influence of miRNA on lncRNA and mRNA regulation is really challenging, since one miRNA can regulate more than one target gene, which consequently affects several pathways in plants [71].

3.2. miRNAs Involved in Response to Long-Term Waterlogging

Although there is more and more research on the involvement of miRNAs in response to abiotic stresses, when it comes to cucumber, the miRNAs were discovered in the context of oxygen deprivation only in the early-stage response to stress [28]. Hence, our research is the first report providing information about the involvement of miRNA in the response

to long-term waterlogging in cucumber. Moreover, we contributed information on the regulation of miRNAs in primed plants.

In this work, 18 libraries of cucumber roots were constructed for non-primed, primed and control plants of the WL-T and WL-S cucumber accessions. The most abundant miRNAs peak size was 24 bp for all experimental groups, which is consistent with results obtained in tolerant cucumber accessions in hypocotyls under waterlogging stress [28]. The same size of miRNA was also detected in cucumber in response to powdery mildew [72]. This may suggest that 24 bp is a typical size for cucumber miRNAs [55].

Bioinformatic analysis revealed only 19 miRNAs with differential expression level in response to long-term waterlogging. All of them were considered to be novel. The obtained miRNAs were analysed in terms of interaction with lncRNAs.

We validated three novel miRNAs and one based on the literature by qRT-PCR assay. The obtained results were in agreement with data provided by small RNA-Seq. Expression of csa-novel_miR1 confirmed the sequencing data, i.e., csa-novelmiR1 was overexpressed in non-primed and primed plants of both accessions. There were no differences in the expression between cucumber accessions, which may suggest that this miRNA is involved in response oxygen deprivation in cucumber regardless of tolerance level. Analysis of target genes prediction depicted that csa-novel_miR1mir1 can regulate the auxin response factor, which is involved in morphological changes of roots [73]. Auxins are one of the most common phytohormones participating in response to hypoxia stress in plants, and the auxin response factor regulates genes involved in auxin signalling pathways through binding to their promoters [74,75].

Csa-novel_miR21 was only expressed in the WL-T accession in the early stage of waterlogging treatment. Target gene prediction revealed that csa-novelmiR21 may regulate gene encoded scarecrow-like protein 15, which participates in root growth under waterlogging, for example in *Brassica napus* [76].

Csa-novel_miR20 was involved in the response to oxygen deprivation in the early stage of both non-primed accessions, its overexpression was also detected after 2 days of the second waterlogging treatment of WL-S. Target gene analysis predicted homeobox-leucine zipper protein ATHB-15 as a potential target of csa-novel_miR20. *ATHB-15* with interaction with miRNA is involved in cell modification, and in consequence, being inhibited by miRNA, causes elongation of roots. It is known that *ATHB-15* is a mir166 target [77], so our results may provide a new specific sequence of mir166 in cucumber.

qRT-PCR validation of miR394 determined up-regulation of this miRNA in early response to the first and second waterlogging treatments of the WL-T accession, which was consistent with the results obtained by Xu [28]. In WL-S, the expression of mir394 was stable in non-primed plants, during the second treatment, after 2 days, expression was inhibited. These results may suggest that mir394 is expressed only in tolerant cucumber accessions, and is involved only in early response to waterlogging.

miRNAs can affect target genes in a positive or negative way, and this may lead to tolerance to abiotic stresses [78].

3.3. Interaction between LncRNAs and miRNAs

In the current research, lncRNAs involved in response to oxygen deprivation were identified, but we also wanted to determine the molecules potentially involved in acquiring tolerance to that stress. As a result, we indicated 71 molecules regulated in non-primed of WL-T and primed plants of WL-S, assuming them to have potential roles in waterlogging tolerance. Among the 71 lncRNAs, TCONS_00019419 was classified as being completely matched to the intron of UDP-glycosyltransferase 1 (Csa4M051380) and TCONS_00020999 exonically overlapped transcription initiation factor TFIID subunit on its opposite strand (Csa4M652030), suggesting that those targets can be regulated by the two identified lncRNAs. Two lncRNAs classified as intergenic, i.e., TCONS_00010998 and TCONS_00003889, were simultaneously up-regulated in non-primed plants of WL-T and primed plants of WL-S, and they could potentially be involved in the gene regulation network, hence their

specific role in the response to hypoxia in cucumber needs to be elucidated. Seven out of 71 lncRNAs can potentially be targeted by miRNAs, resulting in instability of lncRNA. This mechanism has been reported in drought stress in *Solanum lycopersicum* [79], as a response to giberrelin in *Populus tamentosa* [80], and in the flower development of *Cicer arietinum* [81].

LncRNA can act as endogenous target mimics of miRNA, resulting in blocking of the interaction between miRNA and its target gene by, for example, increasing expression [82], and this interaction is considered to be the most crucial in the regulatory gene networks of plants [83]. This mechanism has been reported under heat stress in Chinese cabbage [65], phosphate (Pi) deficiency in *Arabidopsis* [84], and also in species closely related to *Cucumis sativus*, e.g., *Cucumis melo*, in response to powdery mildew [85]. We found that five of the lncRNAs bound the miRNA binding sites of the csa-novel_miRNAs. Additionally, all of those miRNAs were down-regulated under waterlogging stress, meaning that those lncRNAs acted as miRNA decoys, inhibiting their expression.

These interactions showed that the regulatory pathways of those genes are complex under long-term waterlogging stress in cucumber, and an understanding of all of the processes is needed in order to evaluate accessions tolerant to oxygen deprivation. However, information regarding lncRNAs in response to long-term waterlogging in cucumber, to the best of our knowledge, is still not available, so this research provides essential knowledge about the participation of non-coding RNA upon oxygen deprivation in the root zone in cucumber. Additionally, this research made it possible to indicate lncRNAs involved in acquiring tolerance to hypoxia stress via the application of long-term waterlogging priming. The identification of lncRNAs in this research filled a gap in knowledge regarding the involvement of lncRNAs in long-term waterlogging response. Additionally, lncRNA–miRNA interactions were established, which is a big step forward in understanding complex interactions with respect to gene regulation under long-term waterlogging stress in cucumber.

4. Materials and Methods

4.1. Plant Growth and Stress Induction

Seeds of cucumber double haploid lines, DH2, waterlogging tolerant (WL-T), and DH4, waterlogging sensitive (WL-S), with confirmed response to hypoxia stress [7,54], were obtained from a Polish breeding company KHiNO Polan (Krakow, Poland). Plant cultivation and experiment conditions were the same those described in Kęska et al. [7]. Samples for all assays were collected from the same experiment to eliminate the influence of different conditions in order to be able to compare results from each assay with one another. Plants were divided into 3 groups: (1) plants untreated with waterlogging (Ctrl), (2) plants whose root zones were waterlogged for 7 days (1xH, non-primed), and (3) plants waterlogged for a second time after a 14-day recovery period (2xH, primed).

4.2. Sample Collection and RNA Extraction

For small RNA-Seq assay, roots of both cucumber DH lines, WL-T and WL-S, were collected after 7 days of the first hypoxia stress treatment (1xH 7 d) and after 7 days of the second stress induction (2xH 28 d) (Figure 14). For TaqMan^{TM} MicroRNA Assay and lncRNA validation by qRT-PCR, samples were additionally collected after 2 days of both hypoxia stress treatments, in order to detect the expression levels of selected miRNAs and lncRNAs in early phase of stress response. Twelve root samples from plants of control group (Ctrl) were collected at 7 d and 28 d of the experiment (6 from each time-point) and separately divided into 3 independent biological replicates, where one biological replicate was pooled of roots from 4 plants: 2 plants from 7 d and 2 from 28 d. For plants of 1xH and 2xH treatment groups, 3 independent biological replicates were prepared, each pooled with roots from 4 plants. Before freezing in liquid nitrogen, roots were carefully washed in water in order to remove peat substrate. Total RNA extraction from all samples was performed with Direct-zol RNA MiniPrep Plus (Zymo Research, Irvine, CA, USA) following the manufacturer's instructions. To remove DNA contamination, obtained RNA extracts were

treated with 1 U μL^{-1} RNase-free *Dnase* I (Thermo Fisher Scientific, Waltham, MA, USA) and 40 U μL^{-1} of RiboLock RNase Inhibitor (Thermo Fisher Scientific, Waltham, MA, USA). The gel electrophoresis under denaturing conditions was performed to assess RNA quality and quantity. The A260/A280 ratio and RNA integrity number (RIN) were determined by a Bioanalyzer 2100 (Agilent 2100 Bioanalyzer; Agilent Technologies, Palo Alto, Santa Clara, CA, USA) and samples with RIN > 7 were chosen for further analysis.

	2	7	23	28
small RNA-Seq		✓		✓
TaqMan™ MicroRNA Assay	✓	✓	✓	✓
lncRNA in silico identification		✓		✓
lncRNA qRT-PCR validation	✓	✓	✓	✓

Figure 14. A scheme describing the experiment duration and time-points (days) of sample collection for small RNA-Seq assay, TaqMan™ MicroRNA Assay, lncRNA in silico identification and lncRNA validation by qRT-PCR.

4.3. Small RNA Library Preparation, Sequencing and Bioinformatic Analysis of Small RNA Sequencing Data

For small RNA-Seq sequencing, in total, approximately 1 µg total RNA was initially used for BGISEQ-500 library construction. Eighteen libraries (2 cucumber accessions, (i.e., WL-T DH2 and WL-S DH4) × 3 treatments (Ctrl, 1xH, 2xH) × 3 experimental replicates) retaining information about the transcript direction (strand specific) were prepared. Then, these libraries were subjected to next-generation SE50 (single-end 50 bp) NGS sequencing for small RNAs using the BGISEQ-500 platform (BGI, Shenzen, China). The sequencing data for WL-T DH2 (Ctrl, 1xH, 2xH) and WL-S DH4 (Ctrl, 1xH, 2xH) were deposited in the NCBI Sequence Read Archive (SRA, http://www.ncbi.nlm.nih.gov/Traces/sra/) under BioProject No. PRJNA721283.

Analysis of small RNA-Seq data was conducted with CAP-miRSeq [86]. The first stage of the analysis was to check the quality of the reads with FastQC ver. 0.10.1 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). Then the reads were mapped with Bowtie program ver. 0.12.7 (http://bowtie-bio.sourceforge.net/index.shtml) to the cucumber reference genome—ASM407v2 (https://www.ncbi.nlm.nih.gov/assembly/GCF_000004075.2/). The number of reads mapped to individual genes was counted using the HTseq program ver. 0.6.1 (https://htseq.readthedocs.io/en/release_0.10.0/).

The miRDeep2 program [87,88] detected known and novel miRNAs (not present in the database) using data available in the miRBase database ver. 22.1 (http://www.mirbase.org/), reference genome and sequence structural properties. Additionally, the ShortStack program [89] with default settings was applied to confirm predicted novel miRNAs in cucumber. Identified miRNAs were mapped to the cucumber reference genome—ASM407v2 (https://www.ncbi.nlm.nih.gov/assembly/GCF_000004075.2/).

Identification of significantly differently expressed miRNAs (DE-miRNAs) was performed using the edgeR package ver. 3.20.1 [90]. The following comparisons for both cucumbers (WL-S and WL-T) were conducted: 1xH vs. Ctrl, 2xH vs. Ctrl. The miRNAs with FDR $\leq$ 0.05 were considered as differentially expressed between comparisons.

Applying the psRNATarget program (http://plantgrn.noble.org/psRNATarget) [91], potential target sites were detected for differentially expressed miRNAs, matching their sequence with cDNA of the reference genome ASM407v2). The following parameters were implemented, i.e., E $\leq$ 3, maximum energy of unpairing (UPE) the target site was set as

25 kcal, penalty for G:U pair was 0.5 and flanking length around the target site was chosen as 17 nucleotides upstream and 13 nucleotides downstream.

4.4. LncRNA Identification

For the prediction of lncRNAs involved in response to hypoxia stress in cucumber, the assembled transcriptome from PRJNA678740 project deposited in the NCBI Sequence Read Archive (SRA, http://www.ncbi.nlm.nih.gov/Traces/sra/) for Ctrl, 1xH and 2xH of WL-T and WL-S accessions [7] was compared against a reference cucumber transcriptome (ftp://cucurbitgenomics.org/pub/cucurbit/genome/cucumber/Chinese_long/v2) using Cuffcompare ver. 2.2.1 from Cufflinks package implementing the -R and -C options. Then, transcripts were processed according to the following steps: (a) discarding transcripts < 200 nucleotides in length to eliminate miRNA, rRNA, tRNA, snoRNA, siRNA, (b) filtering transcripts containing open reading frames (ORFs) encoding proteins greater than 100 amino acids using TransDecoder [92], (c) elimination of transcripts classified as coding by CPC (coding potential calculator) [93,94], (d) filtering out the sequences described in the Rfam database [95] identified using the BLASTN program (ver. 2.2.26) ($E < 1 \times 10^{-5}$).

Identified lncRNAs were classified according to their genomic context, using Cuffcompare methodology, i.e., '='—complete match of intron chain, 'j'—potentially novel isoform (fragment), at least one splice junction is shared with a reference transcript, 'i'—a transfrag falling entirely within a reference intron, 'o'—generic exonic overlap with a reference transcript, 'u'—unknown, intergenic transcript, 'x'—exonic overlap with reference on the opposite strand.

GO enrichment analysis of the targeted genes of the mRNA:miRNA and mRNA:lncRNA interactions was implemented using the topGO R package ver. 2.38.1 [96].

4.5. Identification of the LncRNA–miRNA Interactions

Identified lncRNAs were checked as to whether they could potentially be targeted by miRNAs or play a role as endogenous target mimicry for miRNAs. An online server psRNATarget was used to predict target sites for miRNAs on lncRNAs with the same parameters set as for miRNAs target gene correlation. TAPIR (http://bioinformatics.psb.ugent.be/webtools/tapir/) [97] was used for prediction of endogenous target mimics (eTMs) with mfe ratio ≥ 0.6 [98]. Networks for the miRNA:lncRNA interactions were built using Cytoscape ver. 3.8.2 (https://cytoscape.org/).

4.6. Quantitative Real-Time PCR (QRT-PCR) Validation of LncRNAs

lncRNA selection for validation by qRT-PCR was based on the results of differential expression analysis of transcripts obtained with the edgeR package ver. 3.20.1 [90]. We randomly selected lncRNAs that, firstly, differentiated accessions from each other after 7 days of waterlogging (1xH) and, secondly, allowed to differ non-primed and primed plants (Table 1). Specific pairs of primers (forward and reverse) for amplification of the selected lncRNAs were designed using Primer3 ver. 3-0.4.0 (http://bioinfo.ut.ee/primer3-0.4.0/) and IDT PrimerQuest (https://eu.idtdna.com). Obtained oligonucleotides were additionally checked with the IDT PrimerQuest program for their physicochemical properties, in order to eliminate the likelihood of the formation of secondary structures (hairpins) as well as heterodimers between the primers of the same pair. List of primers used in the assay can be found in Supplementary Material Table S3. Quantitative real time PCR assay was performed as described in our previous work [7]. Three independent biological replicates, each with three technical replicates, were run for every sample/lncRNA combination. The expression levels of the selected lncRNAs were determined using the ΔΔCt comparative method with the use of two selected reference genes (*Act*, *Tua*) (Supplementary Table S3) and standard curves [99]. The data were then logarithmized ($\log_2$FC) in order to confront the experimentally obtained expression level of the selected lncRNAs with the results of the RNA-Seq analysis.

4.7. Quantitative Real-Time PCR (QRT-PCR) of miRNAs

The expression profiling of selected miRNAs was performed using real-time qPCR. An amount of 2 ng of each RNA sample was processed, strictly following the protocol described in TaqMan™ Small RNA Assay (Publication Number 4364031) using TaqMan™ MicroRNA Reverse Transcription Kit (No 4366596, Applied Biosystems™, Foster City, CA, USA) for cDNA synthesis with miRNA-specific stem-looped RT primers provided in the kit. qRT-PCR reactions were conducted in 96-well plates with 0.67 µL of RT product with TaqMan PCR master mix (No 4304437) and TaqMan probes for each miRNA in a total volume of 10 µL. A QuantStudio™ 3 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) was used to perform the qRT-PCR reactions with U6 as an endogenous control. Three biological replicates were considered, with 3 technical replicates for each of them. The relative expression level of each miRNA was analysed by the ΔΔCt comparative method.

4.8. Statistical Analysis

Comparison between treatments at each time-point and the control condition from the same timepoint was performed. Student's *t*-test with $p < 0.05$ was used to compare changes in relative expression levels of lncRNAs and miRNAs to control conditions as reference. Data are presented as the mean value of three biological replicates ± SD for each treatment and time points. The analysis was performed using Statictica ver. 12 (Statsoft).

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ijms22158197/s1, Supplementary Materials Data S1: Identified lncRNAs, Supplementary Materials Data S2: Differentially Expressed lncRNAs in non-primed 1xH WL-T vs. Ctrl, Supplementary Materials Data S3: Differentially Expressed lncRNAs in primed 2xH WL-T vs. Ctrl, Supplementary Materials Data S4: Differentially Expressed lncRNAs in non-primed 1xH WL-S vs. Ctrl, Supplementary Materials Data S5: Differentially Expressed lncRNAs in primed 2xH WL-S vs. Ctrl, Supplementary Materials Data S6: lncRNAs potentially involved in acquiring tolerance to hypoxia in cucumber, Supplementary Materials Data S7: Differentially Expressed miRNAs in all comparisons, Supplementary Materials Data S8: lncRNAs potentially targeted by miRNAs, Supplementary Table S1: Summary of sequencing results for 18 libraries of miRNA molecules identified for the WL-T and WL-S cucumber accessions, Supplementary Table S2: Complementary pairing of lncRNAs targeted by miRNAs in cucumber under long-term waterlogging, Supplementary Table S3: Primers used in qRT-PCR assay.

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11. Oświadczenia dotyczące udziału kandydata i współautorów

UNIWERSYTET ROLNICZY
im. Hugona Kołłątaja w Krakowie

Wydział Biotechnologii i Ogrodnictwa
Katedra Botaniki, Fizjologii i Ochrony Roślin

Kraków, 14.09.2021

Oświadczenie o współautorstwie

Oświadczam, że w publikacji:

Kołton A, Kęska K, Czernicka M. Selection of Tomato and Cucumber Accessions for Waterlogging Sensitivity through Morpho-Physiological Assessment at an Early Vegetative Stage. Agronomy. 2020; 10(10):1490

mój indywidualny udział w jej powstaniu polegał na:

- sformułowaniu hipotezy badawczej i zaprojektowaniu doświadczeń,
- merytorycznym i technicznym nadzorze nad pomiarami biometrycznymi,
- wykonaniu pomiarów fluorescencji chlorofilu *a,*
- analizie statystycznej wyników i ich wizualizacji,
- interpretacji i dyskusji wyników,
- sformułowaniu wniosków,
- przygotowaniu wstępnej i ostatecznej wersji manuskryptu przed złożeniem do Redakcji,
- odpowiedzi na uwagi recenzentów oraz poprawie zrecenzowanego manuskryptu.

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Katedra Biologii Roślin i Biotechnologii

Oświadczenie o udziale współautorów w publikacji

Oświadczam, że w publikacji *Kołton A, Kęska K, Czernicka M. Selection of Tomato and Cucumber Accessions for Waterlogging Sensitivity through Morpho-Physiological Assessment at an Early Vegetative Stage. Agronomy. 2020; 10(10):1490* mój udział związany był z:

- zaplanowaniem i przeprowadzeniem doświadczenia związanego z badaniem reakcji na stres hipoksji u linii DH ogórka,
- wykonaniem pomiarów biometrycznych i pomiarów fluorescencji chlorofilu *a* u linii DH ogórka,
- zestawieniem otrzymanych wyników zarówno dla pomidora i ogórka oraz przygotowanie danych do analizy metodą skupień,
- przygotowaniem wstępnej i ostatecznej wersji manuskryptu,
- udzieleniem odpowiedzi na uwagi recenzentów.

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Katedra Biologii Roślin i Biotechnologii

Oświadczenie o udziale współautorów w publikacji

Oświadczam, że w publikacji *Kęska K, Szcześniak MW, Makałowska I, Czernicka M. Long-Term Waterlogging as Factor Contributing to Hypoxia Stress Tolerance Enhancement in Cucumber: Comparative Transcriptome Analysis of Waterlogging Sensitive and Tolerant Accessions. Genes (Basel). 2021 Jan 28;12(2):189* mój udział związany był z:

- współudziałem w opracowaniu metodologii doświadczenia,
- przeprowadzeniem doświadczenia szklarniowego,
- wykonaniem analiz laboratoryjnych (izolacja RNA, analiza ekspresji genów metodą qRT-PCR),
- wykonaniem analizy statystycznej wyników,
- wykonaniem części analiz bioinformatycznych danych RNA-Seq,
- opracowaniem, interpretacją, wizualizacją oraz dyskusją uzyskanych wyników,
- przygotowaniem wstępnej i ostatecznej wersji manuskryptu,
- udzieleniem odpowiedzi na uwagi recenzentów oraz poprawą manuskryptu po recenzji.

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UNIWERSYTET IM. ADAMA MICKIEWICZA W POZNANIU
Wydział Biologii

Poznań, 8 września 2021 r.

Oświadczenie o udziale współautorów w publikacji

Oświadczam, że w publikacji *Kęska K, Szcześniak MW, Adamus A, Czernicka M. Waterlogging-Stress-Responsive LncRNAs, Their Regulatory Relationships with miRNAs and Target Genes in Cucumber (Cucumis sativus L.). Int J Mol Sci. 2021 Jul 30;22(15):8197* mój udział dotyczył merytorycznego i metodycznego wsparcia podczas analiz bioinformatycznych.

.....

dr hab. Michał Szcześniak, prof. UAM

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UNIWERSYTET IM. ADAMA MICKIEWICZA W POZNANIU
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Wydział Biologii

Poznań, 8.09.2021

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- współudziałem w opracowaniu metodologii doświadczenia,
- przeprowadzeniem doświadczenia szklarniowego,
- wykonaniem analiz laboratoryjnych (izolacja RNA, analiza ekspresji lncRNA i miRNA oraz genów targetowych metodami qRT-PCR i TaqMan^{TM} Small RNA Assay),
- wykonaniem analiz bioinformatycznych danych miRNA-Seq,
- identyfikacją lncRNA na bazie danych RNA-Seq ogórka,
- wykonaniem analizy statystycznej wyników,
- opracowaniem, interpretacją, wizualizacją oraz dyskusją uzyskanych wyników,
- przygotowaniem wstępnej i ostatecznej wersji manuskryptu,
- udzieleniem odpowiedzi na uwagi recenzentów oraz poprawą manuskryptu po recenzji.

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Wydział Biologii

Poznań, 8 września 2021 r.

Oświadczenie o udziale współautorów w publikacji

Oświadczam, że w publikacji *Kęska K, Szcześniak MW, Makałowska I, Czernicka M. Long-Term Waterlogging as Factor Contributing to Hypoxia Stress Tolerance Enhancement in Cucumber: Comparative Transcriptome Analysis of Waterlogging Sensitive and Tolerant Accessions. Genes (Basel). 2021 Jan 28;12(2):189* mój udział dotyczył merytorycznego i metodycznego wsparcia podczas analiz bioinformatycznych.

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Katedra Biologii Roślin i Biotechnologii

Oświadczenie o udziale współautorów w publikacji

Oświadczam, że w publikacji *Kęska K, Szcześniak MW, Adamus A, Czernicka M. Waterlogging-Stress-Responsive LncRNAs, Their Regulatory Relationships with miRNAs and Target Genes in Cucumber (Cucumis sativus L.). Int J Mol Sci. 2021 Jul 30;22(15):8197* mój udział związany był krytyczną recenzją napisanego manuskryptu.

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Oświadczenie o udziale współautorów w publikacji

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