REGISTRATION FORM (BASIC DATA)

1a. Details of applicant

Name, first name, title(s): Prof. Dr. J. C. M. (Sjef) Smeekens
Male
Institution: Utrecht University
Department: Molecular Plant Physiology, Institute of Environmental Biology, Utrecht University
Position: **X** Professor O Associate professor (UHD) O Assistant professor (UD)
Permanent position: **X** Yes O No, end date contract:

1c. Proposed PhD candidate

Name, first name, title(s): Van der Woude, Lennard, BSc (Male)

2. Title of research proposal

Unraveling the molecular mechanisms underlying high ambient temperature acclimation in plants using the novel growth regulator Heatin

3. Summary of research proposal

(scientific summary in English, max 250 words)

High ambient temperature is most detrimental to plants and crop yield. Food security is therefore threatened in the current era of global warming and an increasing world population. Plants respond to high ambient temperatures by several architectural adaptations, collectively called the High Temperature Acclimation Response (HTAR). Despite the important societal and economic impact of these adaptations, the molecular mechanisms underlying HTAR and thermosensing are hardly understood.
I have identified a novel growth regulator that enhances HTAR using a chemical genetics screen. This novel growth regulator, named Heatin, does not affect plant growth under control temperatures. In this project, I propose to use Heatin to understand the mechanisms underlying HTAR. I propose to identify the plant protein targets of Heatin and to uncover genetic networks targeted by Heatin. A transcriptome analysis will reveal genes regulated by Heatin. In addition, a genome-wide association study that links genomic loci to the Heatin response will be performed. This information will be combined with the transcriptomics results. Genes that are transcriptionally regulated by Heatin and map to genes/genomic loci associated with Heatin sensitivity, and the genes coding for Heatin's protein targets, will be studied in detail to understand their role in HTAR. Such analysis will deliver novel insights in the molecular networks how plants regulate HTAR. This knowledge will facilitate breeding programs aimed to develop crops that maintain high yields in a warming climate.

4. Main field of research

(state code and field of research; http://www.nwo.nl/financiering/nwo-disciplinecodes):
Biology; 22.50.00 Botany

5. Summary for the general public

(please provide in 100 words a title and summary for the general public, preferably in Dutch)

Hoe planten zich aanpassen aan hoge temperatuur.

Klimaatopwarming leidt tot significante verliezen in gewas opbrengst. Planten kunnen hun groei en ontwikkeling echter aanpassen aan hoge temperatuur om de schadelijke effecten te beperken. De moleculaire mechanismes die daaraan ten grondslag liggen zijn nog onbekend. Recent is een nieuwe chemische stof gevonden, Heatin genaamd, die de aanpassing van planten aan hoge temperatuur stimuleert. In dit project gaan de onderzoekers Heatin inzetten om de mechanismes te ontrafelen waarmee gewassen zich aanpassen aan hoge temperatuur. Dit is van belang voor de ontwikkeling van robuuste gewassen die bestand zijn tegen klimaatopwarming.

DESCRIPTION OF THE PROPOSED RESEARCH

6. Description of the proposed research

Max. 4 pages, including figures, excluding literature references. Include details of objectives, scientific approach, impact, innovative aspects, and literature references (include full bibliographical details).

Primary aim and novel aspects of the project

Even a small increase in ambient temperature severely affects growth and development throughout the life of plants and leads to major decreases in crop yield [1,2]. To mitigate the effects of high temperatures, plants can adjust their growth and morphology [3-6]. This High Temperature Acclimation Response (HTAR) includes embryonic stem (hypocotyl) elongation, elongation of the leaf stalks (petioles) and upward leaf movement (hyponasty). Together, this suite of traits enhances evaporative cooling [7,8]. However, it is largely unknown how plants perceive and transmit high temperature signals and activate high temperature acclimation.

Numerous mutant screens have resulted in the identification of genes controlling growth under different environmental conditions in the model plant *Arabidopsis thaliana.* Such classical genetics approaches have thus far not uncovered major thermosensory pathways. Given the importance of ambient temperature sensing and acclimation, several redundant pathways have likely evolved that cannot easily be dissected by classical genetics [9,10]. To circumvent this problem of redundancy, I performed a high-throughput chemical genetic screen to isolate novel compounds that enhance plant heat acclimation and identified a compound named 'Heatin'. **Heatin is a positive regulator of hypocotyl elongation under high temperature conditions but does not affect growth under control temperatures. In this project Heatin will be used to gain mechanistic understanding of the molecular networks underlying plant acclimation to high ambient temperature.** I propose to:

i) Characterize Heatin-mediated HTAR and optimize the chemical structure of Heatin to enhance its effects on HTAR in *Arabidopsis* and crops.
ii) Identify the molecular and transcriptional networks that are affected by Heatin activity.
iii) Determine how Heatin's target proteins and regulated genes control HTAR in *Arabidopsis* and crops.

This project will give much needed novel insights into how plants acclimate to changing environmental conditions, specifically high ambient temperature. This knowledge is important for improving thermotolerance and maintaining yield of important crops exposed to current rapid climate change.

Background and unpublished findings

Climate models predict that the earth's temperature will rise by 0.8-4^{o}C in the coming decades [11]. This rise severely decreases crop yields [1,2] and conflicts with the need of doubling food production in the next ~50 years to feed a growing and more demanding world population. Plants can mitigate the impact of high ambient temperatures by inducing a set of morphological adaptations, called the High Temperature Acclimation Response (HTAR) [6], which allows optimal growth under detrimental temperature conditions.

More than a decade ago, Gray *et al.* [3] elegantly demonstrated that *Arabidopsis thaliana* is an excellent model to study acclimation responses to high temperature. These authors showed that transfer of seedlings to higher ambient temperatures (from 20℃ to 29℃) results in an incr ease in hypocotyl length (Fig. 1a) and that this process depends on the phytohormone auxin. Later studies added among other traits; increased petiole length and hyponasty (upward leaf movement) (Fig. 1b, c) to the palette of morphological adaptations induced in response to high temperatures [4,5,7]. Together, this results in an open rosette structure which enhances leaf cooling capacity [7,8]. Manipulating HTAR experimentally or trough breeding can therefore greatly contribute to safeguarding food security.

It remains elusive how plants sense ambient high temperature and what molecular processes transduce temperature signals into relevant acclimation responses. Temperature affects almost every process in the plant and likely several parallel and redundant temperature sensing mechanisms evolved [10]. Basic and vital processes such as enzyme activities and membrane composition and fluidity are temperature-dependent [6,9]. Therefore, every process in the plant can potentially contribute to thermosensing. This proposed redundancy hampered isolating key genes involved in HTAR and only recently our understanding of the molecular pathways involved in thermosensing and HTAR advanced [6,10]. In a breakthrough paper, Kumar and Wigge [12] demonstrated that a specific histone H2A variant (H2A.Z) contributes to thermosensing. Mutants in *ACTIN-RELATED PROTEIN 6* (*ARP6*), disturbed in H2A.Z positioning on the chromatin, show exaggerated HTAR traits. Additionally, Koini *et al.* [4] demonstrated that full induction of high temperature-mediated hypocotyl and petiole elongation, as well as hyponastic growth, require the bHLH transcription factor *PHYTOCHROME INTERACTING FACTOR 4* (*PIF4*). Interestingly, *pif4* mutants display severely reduced HTAR, while mutants in homologous *PIF* family members did not abolish responsiveness to high temperature. Analysis of high temperature-induced genes demonstrated that *PIF4* does not affect general temperature-sensing mechanisms. Based on this it was concluded that *PIF4* is specifically required for HTAR in *Arabidopsis* [4,13]. Recently, PIF4 was shown to directly stimulate auxin biosynthesis by binding the promoters of genes coding for auxin biosynthesis enzymes in a temperature-dependent manner [14,15]. Franklin *et al*. [14] demonstrated that *PIF4*-mediated expression of *SMALL AUXIN UP-RNA* genes (*SAUR19-24*) contributes to high temperature-induced hypocotyl elongation. Finally, in addition to auxin, the phytohormones gibberellins and brassinosteroids are important for hypocotyl elongation under high temperature [5,13].

I decided to use a chemical genetics approach to identify molecular networks involved in HTAR. The advantage of this strategy is that the problem of genetic redundancy is circumvented, which hampers classical genetics approaches [16,17]. Chemical genetics originated as a tool for drug discovery and has now been successfully applied in plant research several times, with as most notable example the identification of the highly redundant PYRABACTIN RESISTANCE 1 (PYR1) family of phytohormone ABA receptors [18].

I performed a high-throughput chemical genetics screen to identify growth regulators that enhance high temperature acclimation in *Arabidopsis thaliana* as part of my second MSc-project, in the group of Dr. S. Robert at the Swedish University for Agricultural Sciences. A large and diverse library of small aromatic compounds (Chembridge; www.chembridge.com) was screened for the ability to enhance high temperature acclimation. For screening, I used high temperature-induced hypocotyl elongation as this represents a practical and reliable assay for HTAR.
8000 compounds were initially screened and subsequently the effects of candidate compounds were confirmed. Because auxin is a potent inducer of HTAR, candidate compounds that were structurally related to auxins were omitted. To further exclude auxins, the 10 remaining candidate compounds were tested for their ability to induce the typical auxin-induced agravitropic response, in which plants lose track of the direction of gravity and grow in random orientation. Moreover, the ability of each compound to activate auxin signalling was assayed by histochemical staining using a transgenic line driving the β-glucuronidase gene from the auxin-inducible synthetic *DR5* promoter [19].
Next, a dose-response assay was performed under control and high temperature conditions on the Col-0 wild type and *pif4-2* mutant genotype. This was done to identify the most promising compound for follow-up studies and to position the compound's effect upstream, parallel or downstream of PIF4 action. These selection criteria led to the identification of 'Heatin' (C16H23N3O2). **Heatin strongly enhances elongation growth in a high temperature- and dose-dependent manner, independent of auxin, in the wild type Col-0 background, but cannot rescue the abolished high temperature-induced hypocotyl elongation phenotype of the *pif4-2* mutant (fig. 1d,e). This suggests that Heatin targets molecular component(s) upstream of the PIF4/auxin or in a parallel novel signalling pathway.**

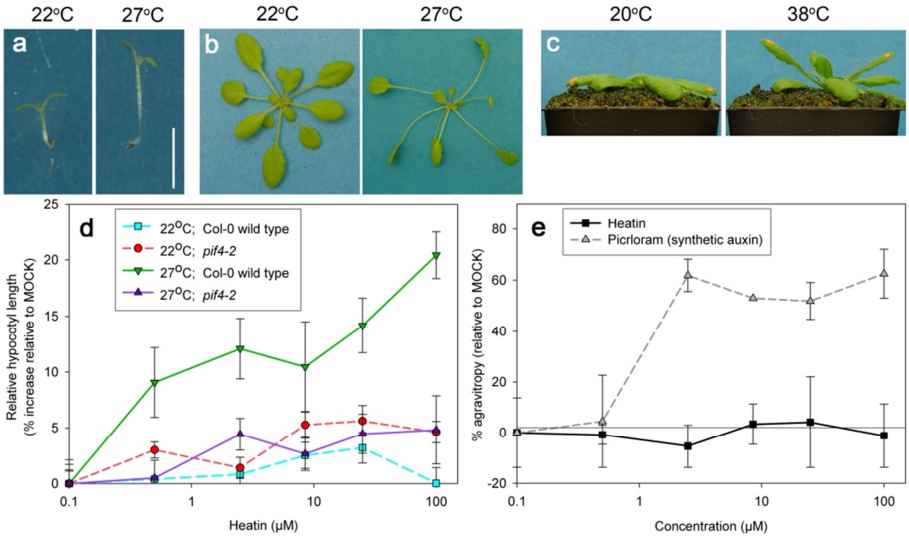

Figure 1: High temperature acclimation is stimulated by Heatin. a) Typical high temperature-induced hypocotyl elongation in 7d-old seedling grown at 27ºC compared to 22ºC. Scale bar is 0.5 cm. **b)** Characteristic open rosette morphology and **c)** hyponasty (upward oriented leaves) of warm-grown plants (30d-old at 22ºC and 27ºC (**b**) and 20ºC and 38ºC (**c**). **d)** Dose-response effects of Heatin application on hypocotyl elongation in Col-0 wild type (green line/triangle down, blue line/squares) and *pif4-2* mutant (red line/circles, purple/triangle up) at control (22ºC;dashed lines) and high temperature (27ºC; closed lines). **Note that Heatin is a temperature-specific agonist of hypocotyl elongation and requires PIF4 activity. e)** Heatin does not induce agravitropy (random growth orientation), indicating that Heatin does not have auxin activity. Compare the response to Heatin (Col-0; 27ºC) to that of application of the synthetic auxin Picloram. Error bars represent SD.

In this project I propose to use Heatin to unravel the molecular mechanisms and genetic networks underlying plant acclimation to high ambient temperature. First, the chemical and bioactive properties of Heatin will be assessed by a structure-activity relation study and Heatin's effect on other HTAR traits (hyponasty, elongation growth etc) will be explored. Next, Heatin will be used as a tool to identify novel genes and proteins involved in high ambient temperature acclimation. To this aim, I will use a biochemical as well as a genetic approach. In the biochemical approach the direct targets of Heatin will be determined by affinity purification of proteins interacting with Heatin, followed by their identification using Liquid chromatography–mass spectrometry (LC-MS) [17]. In the genetic approach, first the effects of Heatin on the *Arabidopsis* transcriptome will be assessed by microarray analysis. Next, I will use Genome-Wide Association mapping (GWA) to detect candidate Single Nucleotide Polymorphisms (SNPs) affecting Heatin-mediated high temperature-induced hypocotyl elongation in natural *Arabidopsis* accessions. Overlaying the transcriptome data with the SNPs detected by the GWA-mapping will result in the identification of stringently selected genes that potentially contribute to HTAR. The most promising genes identified by the genetic approaches and the genes coding for Heatin's direct protein targets, identified by the biochemical approach, will be subjected to detailed functional molecular characterization. This will result in novel understanding of how these genes and their proteins affect HTAR. Homologous genes will then be identified in crops and tested for their contribution to HTAR and how this affects crop yield when facing high ambient temperatures. In addition, the potential of applying Heatin to prime crops for high temperature stress will be assessed. This knowledge is highly valuable for the plant breeding industry that needs more thermotolerant crops that maintain a high yield under current rapid global climate change.

Objective/Chapter 1: Characterizing Heatin's effects on high temperature acclimation
Aims: i) To characterize the effect of Heatin on HTAR in *Arabidopsis.* ii) To position Heatin in the framework of existing knowledge of HTAR-regulation.
1a) The effects of Heatin on HTAR will be thoroughly analyzed in *Arabidopsis*. Heatin will be applied to Col-0 wild-type plants and *pif4-2* mutants, grown under control and high temperatures (22℃ and 27℃) and the effect of Heatin on HTAR phenotypes will be quantified. These include hyponasty (upward leaf movement), petiole elongation and floral induction. In parallel, the effect on Heatin on several phenotypes that are indicators/proxies of traits related to quality and quantity of harvestable products will be assessed under control and high temperatures. This will include flowering time (total leaf number, days-to-flowering), number of viable flowers, silique (fruit) length, number of seeds per silique, branching number and seed yield per plant. Phenotyping will be done following standard protocols that are routinely performed in the Molecular Plant Physiology (UU) laboratory. Assessing the effect on floral induction will be done in cooperation with Dr. Martijn Fiers (WUR), who developed a luciferase-based screening platform to test the effect of chemicals on floral induction. This platform is available for this project. The information obtained in this Objective/Chapter is needed for Objectives/Chapters 1b, 2, 4 and 5.
1b) To position Heatin in the framework of the current knowledge of HTAR regulation, the effect of Heatin on hypocotyl elongation and other relevant HTAR traits identified in objective 1a will be tested in the presence of phytohormones (*e.g.* Auxin, Gibberellins, Brassinosteroid, Abscisic Acid) and pharmacological agents interfering with their biosynthesis/activity/transport (*e.g.* NPA, TIBA, Brassinozol, Brassinolide, Fluridone, Paclobutrazol). Moreover, quantitative Real-Time PCR analysis will be performed on genes involved in HTAR [3-5,12-15]. This will include the *PIF* family members, high temperature-induced auxin synthesis (*TAA1, YUC8* and *CYP79B2*) and signaling (*SAUR19-24, IAA19, IAA29* etc) genes as well as *ARP6.* To assess if Heatin interferes with general thermosensing, known thermoresponsive genes, including *HSP70* and *HSFA2,* will also be tested. The expression of genes will be assayed at various time points after Heatin/mock and high/control temperature treatment, to time Heatin action. This information is critical for Objective/Chapter4a and 5a. Next, dependent on the outcome of these qRT-PCRs, the effect of Heatin on HTAR will be tested in specific mutants disturbed in, for instance, the above mentioned hormone signaling/biosynthesis genes. These mutants are mostly already available at Utrecht University or will be obtained through the common *Arabidopsis* seed stock-centers (NASC/ABRC; www.arabidopsis.info) or through authors who described them.

Objective/Chapter 2: Structure-activity relation study to optimize Heatin's bioactivity and amending the molecule for biochemical purification of Heatin's protein targets
Aims: i) Optimizing the bioactivity of Heatin. ii) Identify the chemical characteristics of Heatin that are (in)dispensable for its bioactivity. iii) Chemically amending Heatin for purification of its protein targets.
2a) Several commercially available compounds exist that are structurally analogous to Heatin (C16H23N3O2). Related compounds will be identified through existing databases such as Chemspider (http://www.chemspider.com), PubChem (https://www.ncbi.nlm.nih.gov/pccompound) and Chembridge. These compounds will be ordered at the Chembridge company (Hit2Lead.com). Next, their effects on high temperature-induced hypocotyl elongation and other HTAR traits, as well as previously established marker genes for Heatin action (Objective/Chapter 1a,1b), will be quantified. This structure-activity relation study will uncover which chemical groups are essential and which can be substituted without compromising Heatin's bioactivity.
2b) Using the structure-activity information obtained in Objective/Chapter 2a, an optimal functioning next-generation Heatin compound (Heatin-II) will be synthesized in the laboratory of Prof. R. Pieters and Dr. N. Martin (Department of Medicinal Chemistry & Chemical Biology, UU), who agreed on participating in this project. Additionally, Heatin-derived compounds will be synthesized in this laboratory, optimized for identification of Heatin's direct protein targets. This will be done by replacing chemical groups that are dispensable for Heatin bioactivity with a photo-reactive binding group and a biotin marker-molecule, or by an alkyne minitag, which can be subsequently coupled to the photoreactive and biotin group through click-chemistry, if more feasible [20]. This modified compound (called Heatin-II-Biotin) will be instrumental in detection of Heatin's target proteins (Objective/Chapter 3a). Before proceeding, the effect of the two modified Heatin compounds (Heatin-II and Heatin-II-Biotin) will be confirmed by comparing their bioactivity with the original Heatin. To this aim, essentially key-phenotyping experiments and expression analyses described in Objective/Chapter 1a and 1b will be repeated.

Objective/Chapter 3: Biochemical purification and identification of Heatin-II target proteins
Aims: Identification of the direct protein targets of Heatin-II.
Heatin-II-Biotin (and mock) will be incubated with protein extracts isolated from control and high temperature-treated *Arabidopsis* Col-0 plants. The protein samples will be exposed to UV-radiation to activate the photo-reactive group. This results in the formation of covalent bonds between Heatin-II and its target proteins. Next, commercially obtained Streptavidin-coupled magnetic Dynabeads® (Life technologies, Ltd) will be added to capture the Heatin-II-Biotin attached to the Heatin-target protein. The biotin/streptavidin-Heatin-II-protein complex will be pulled-down using a magnet, washed and eluted using an on-bead tryptic digest according to standard protocols. Subsequently, the pulled-down proteins will be identified by quantitative Liquid Chromatography–Mass Spectrometry (LC-MS). As a control, Heatin-II, without the biotin and photo-reactive group modifications, will be titrated in different concentrations into independent samples. This will allow me to discriminate true Heatin-II-binding targets from false positives. True targets will decrease with increasing concentrations of added Heatin-II due to competitive binding. The quantitative LC-MS signal of these true binding targets, but not of non-specific pulled down proteins, will decrease with increasing concentrations of unmodified Heatin-II. The genes encoding for the identified proteins, will be studied in functional and molecular detail (Objective/Chapter 5) to unravel how these contribute to high temperature acclimation.

I will perform the pull down and LC-MC experiments in the lab of Dr. R. van der Hoorn (Oxford University, UK). He and his co-workers are experts on detecting protein targets of small molecules in plants [17,20,21]. Dr. van der Hoorn already agreed on participating in this project and on hosting me in his lab for the duration of the experiments.

Objective/Chapter 4: Identification of genes contributing to Heatin-II-mediated HTAR

Aims: i) Identify the effects of Heatin-II on the transcriptome. ii) Identify novel genes and natural genetic variants (Single nucleotide polymorphisms; SNPs), that contribute to Heatin-II-mediated HTAR. iii) Stringent selection of candidate genes for functional and molecular study of the molecular networks underlying plant acclimation to high temperature.

4a) The effects of Heatin-II on the *Arabidopsis* transcriptome will be assessed. Hypocotyl tissues, or other tissues if more feasible depending on the outcome of Objective 1a, will be harvested of Col-0 seedlings grown at 22^{o}C and 27^{o}C. The exact harvest time will depend on the outcomes of the time-course study of marker gene expression in Objective/Chapter 1b. The new Titan ST1.0 Affymetrix microarrays will be used for transcriptome analysis. Hybridization and scanning of the arrays will be out-sourced to a company (*e.g.* ServiceXS). Data will be analysed with existing software (R freeware) to detect differentially expressed genes. Subsequently, the affected biological processes will be identified using bioinformatics approaches (*e.g.* Mapman analysis).

4b) Large numbers of natural *Arabidopsis* accessions, from diverse natural environments, have been thoroughly genotyped or sequenced [22,23]. This enables identification of small differences in the DNA sequence (single nucleotide polymorphisms; SNPs and insertions and deletions) contributing to natural phenotypic variation in a trait of interest. Genome Wide Association (GWA) mapping will be performed to identify natural occurring SNPs that affect Heatin-II mediated HTAR. I will quantify the effect of Heatin-II on high temperature-induced hypocotyl elongation in an existing panel of 349 natural *Arabidopsis* accessions [22], which have been extensively genotyped (250k SNPs). 15 seedlings of each accession will be grown under high (27℃) and control (22℃) temperatures, in the presence and absence of Heatin-II on standard MS-growth medium. Plates will be scanned and hypocotyl lengths will be semi-automatically measured using ImageJ image analysis freeware. Candidate SNPs associated to Heatin-II-mediated hypocotyl elongation will be identified using the EMMAX mapping algorithm which has been successfully used in the host group to identify SNPs associated to upward leaf movement in response to high temperatures (M. van Zanten, *unpublished*).

4c) The data sets of objectives 4a and 4b will be combined to make a stringent selection of genes and genetic networks that control Heatin-II-mediated HTAR. I will make a priority list of candidate genes of only those genes that are differentially affected by Heatin-II at high temperature but not at control temperature. These genes will be considered for further analysis (objective 5). The prioritized genes will be independently validated by qRT-PCR. This objective is expected to uncover novel genes and genetic networks that have not previously been associated with HTAR and may reveal novel thermosensory pathways.

This Objective/Chapter requires extensive bioinformatics expertise. This is present in the hosting group (Dr. M. van Zanten, Dr. J. Hanson, at the neighbouring group of Dr. B. Snel (UU) and through existing collaborations of Dr. M. van Zanten with the group of Prof. F. van Eeuwijk, Biometris/WUR). However, statistical and bioinformatics courses from EPS will be integrated into the projects educational goals, to increase my knowledge on this subject.

Objective/Chapter 5: Functional and mechanistic characterization of how Heatin-II-affected genes contribute to HTAR in *Arabidopsis* and relevant crop species/varieties

Aims: i) Obtain novel mechanistic understanding of how genes affected by Heatin-II contribute to HTAR. ii) Identify novel HTAR/themosensory mechanisms. iii) Assess if Heatin-II-affected genes isolated from *Arabidopsis* can contribute to improvement of HTAR and of harvestable product quality/quantity in important crop species/varieties.

5a) Many tools are available to efficiently characterize *Arabidopsis* genes on the molecular physiological level. In this objective the genes coding for the direct protein targets of Heatin-II (Objective/Chapter 3) as well as the most promising candidate genes controlling Heatin-II-mediated HTAR (Objective/Chapter 4c) will be studied in molecular detail. For example, available bioinformatics tools (www.arabidopsis.org) will be consulted, knock-out mutants will be obtained from existing resource centres (*e.g.* http://signal.salk.edu/cgi-bin/tdnaexpress) and overexpressing lines will be generated. Depending on the nature of the genes, crosses will be made to mutants disturbed in *e.g.* hormonal signalling pathways involved in HTAR, such as auxin-related mutants (Chapter/Objective 1). In all these (generated) lines a selection of HTAR traits as well as expression of identified marker genes (Objective/Chapter 1, 4) will be assayed under control (22^{o}C), and high temperature (27^{o}C), conditions in the presence and absence of Heatin-II.

5b) Heatin-II effects on relevant commercial crops will be evaluated. First, the effect of Heatin-II on HTAR will be assessed in a selection of crop species and varieties. The choice of crops for this objective will depend on the outcomes of a project that is currently running at HAS-Hogeschool in 's Hertogenbosch (Dr. E. Clerkx and Dr. M. van Zanten, *unpublished*) in which the extend of HTAR is explored in 120 economically relevant crop-species/varieties. The crop species/varieties that exhibit HTAR will be treated with Heatin-II and the compound's effect on HTAR will be quantified. This will indicate if Heatin-II has the potential to be used commercially by farmers to either prime crops for upcoming high ambient temperatures in the greenhouse/field or to mitigate the negative effects of persisting high ambient temperatures. Next, homologues of genes confirmed to contribute to Heatin-II-mediated enhancement of HTAR in *Arabidopsis* (Objective/Chapter 5a) will be identified in crop species/varieties that display HTAR and are sensitive to Heatin-II either based on the many genome sequences that are available or through cloning and sequencing the homologous genes using degenerate primers designed on conserved parts of the DNA sequence. The effect of Heatin-II on the transcription of these genes under different ambient temperatures will be assessed to evaluate if these genes potentially contribute to HTAR in these crop species/varieties by the same mechanisms as in *Arabidopsis*. Such genes represent important breeding markers for thermotolerance improvement and can be directly used by plant breeders to generate thermotolerant varieties, which maintain yield under the restriction of global warming. This objective will be conducted in collaboration with BEJO zaden BV, Warmenhuizen, who support this proposal and intent to provide *in kind* contribution (see the letter-of-support attached to this proposal).

References

1. Battisti DS, Naylor RL (2009) Historical warnings of future food insecurity with unprecedented seasonal heat. *Science* 323: 240-244
2. Lobell DB, Gourdji SM (2012) The influence of climate change on global crop productivity. *Plant Physiol*. 160: 1686-1697
3. Gray WM, Ostin A, Sandberg G, Romano CP, Estelle M (1998) High temperature promotes auxin mediated hypocotyl elongation in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA.* 95: 7197–7202
4. Koini MA, Alvey L, Allen T, Tilley CA, Harberd NP, Whitelam GC, Franklin KA (2009) High temperature-mediated adaptations in plant architecture require the bHLH transcription factor PIF4. *Curr. Biol.* 19: 408-413
5. Van Zanten M, Voesenek LACJ, Peeters AJM, Millenaar FF (2009) Hormone- and light-mediated regulation of heat-induced differential petiole growth in *Arabidopsis thaliana. Plant Physiol.* 151: 1446-1458
6. Van Zanten M, Bours R, Pons TL, Proveniers MCG (2013) Plant acclimation and adaptation to warm environments. In; *Temperature and Plant Development*, (Eds. Franklin KA & Wigge PA), Wiley-Blackwell publishers, *in press*
7. Crawford AJ, McLachlan DH, Hetherington AM, Franklin KA (2012) High temperature exposure increases plant cooling capacity. *Curr. Biol.* 22: 396-397
8. Bridge LJ, Franklin KA, Homer ME (2013) Impact of plant shoot architecture on leaf cooling: a coupled heat and mass transfer model. *J. R. Soc. Interface* 10
9. Penfield S (2008) Temperature perception and signal transduction in plants. *New Phytol.* 179: 615-628
10. Wigge PA (2013) Ambient temperature signalling in plants. *Curr. Opin. Plant Biol.* 23: *in press*
11. IPCC (2013) Climate change 2013: The physical science basis. Contribution of working group I to the fifth assessment report of the Intergovernmental Panel on Climate Change. Stocker T, Dahe Q, Plattner G-K (lead eds.). *Cambridge University Press,* Cambridge, UK.
12. Kumar SV, Wigge PA (2010) H2A.Z-containing nucleosomes mediate the thermosensory response in *Arabidopsis*. *Cell* 140:136-147
13. Stavang JA, Gallego-Bartolomé J, Gómez MD, Yoshida S, Asami T, Olsen JE, García-Martínez JL, Alabadí D, Blázquez MA (2009) Hormonal regulation of temperature-induced growth in *Arabidopsis*. *Plant J.* 60: 589-601
14. Franklin KA, Lee SH, Patel D, Kumar SV, Spartz AK, Gu C, Ye S, Yu P, Breen G, Cohen JD, Wigge PA, Gray WM (2011) Phytochrome-interacting factor 4 (PIF4) regulates auxin biosynthesis at high temperature. *Proc. Natl. Acad. Sci USA.* 108: 20231-20235
15. Sun J, Qi L, Li Y, Chu J, Li C (2012) PIF4-Mediated activation of YUCCA8 expression integrates temperature into the auxin pathway in regulating *Arabidopsis* hypocotyl growth. *PLoS Genet.* 8: e1002594
16. McCourt P, Desveaux D (2010) Plant chemical genetics. *New Pythol.* 185: 15-26
17. Tóth R, Van der Hoorn RA (2010) Emerging principles in plant chemical genetics. *Trends Plant Sci.* 15: 81-88
18. Park SY, Fung P, Nishimura N, Jensen DR, Fujii H, Zhao Y, Lumba S, Santiago J, Rodrigues A, Chow TF, Alfred SE, Bonetta D, Finkelstein R, Provart NJ, Desveaux D, Rodriguez PL, McCourt P, Zhu JK, Schroeder JI, Volkman BF, Cutler SR (2009) Abscisic acid inhibits type 2C protein phosphatases via the PYR/PYL family of START proteins. *Science* 324: 1068-1071
19. Ulmasov T, Murfett J, Hagen G Guilfoyle TJ (1997) Aux/IAA proteins repress expression of reporter genes containing natural and highly active synthetic auxin response elements. *Plant Cell* 11:1963-1971
20. Kaschani F, Verhelst SH, van Swieten PF, Verdoes M, Wong CS, Wang Z, Kaiser M, Overkleeft HS, Bogyo M, van der Hoorn RA (2009) Minitags for small molecules: detecting targets of reactive small molecules in living plant tissues using 'click chemistry'. *Plant J.* 57: 373-385
21. Lenger J, Kaschani F, Lenz T, Dalhoff C, Villamor JG, Köster H, Sewald N, van der Hoorn RA (2012) Labeling and enrichment of *Arabidopsis thaliana* matrix metalloproteases using an active-site directed, marimastat-based photoreactive probe. *Bioorg. Med. Chem.* 20: 592-596
22. Baxter I, Brazelton JN, Yu D, Huang YS, Lahner B, Yakubova E, Li Y, Bergelson J, Borevitz JO, Nordborg M, Vitek O, Salt DE (2010) A coastal cline in sodium accumulation in *Arabidopsis thaliana* is driven by natural variation of the sodium transporter AtHKT1;1. *PLoS Genet.* 11: e1001193
23. Bergelson J, Roux F (2010) Towards identifying genes underlying ecologically relevant traits in *Arabidopsis thaliana*. *Nature Rev. Genet.* 11: 867-879
24. Mittler R, Kim Y, Song L, Coutu J, Coutu A, Ciftci-Yilmaz S, Lee H, Stevenson B, Zhu JK (2006) Gain- and loss of-function mutations in Zat10 enhance the tolerance of plants to abiotic stress. *FEBS Lett.* 580: 6536-6542
25. Barnabás B, Jäger K, Fehér A (2008) The effect of drought and heat stress on reproductive processes in cereals. *Plant Cell Environ.* 31: 11-38
26. Parent B, Tardieu F (2012) Temperature responses of developmental processes have not been affected by breeding in different ecological areas for 17 crop species. *New Phytol.* 194: 760-774

7. Timetable of the project

Give a practical timetable over the grant period, max. 0.5 page.

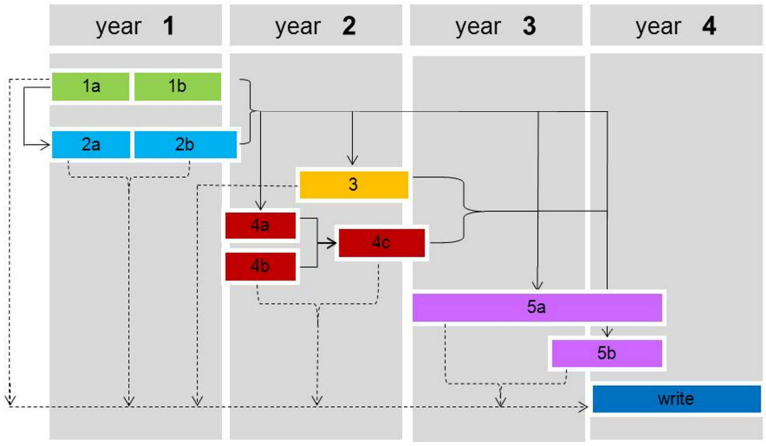

Figure 2: Time schedule and flow scheme of the intended program. The five objectives/chapters are tightly integrated and experimental setup and/or results generated in each objective will link to subsequent objectives (closed lines/arrows). Each objective also yields stand-alone data, which can be followed up on in case of the unexpected delay of one of the objectives. Each of the five objectives will result in a chapter in the final PhD dissertation (dashed lines).

The proposed program will be conducted in four years (Figure 2) resulting in the writing of a PhD dissertation. The five intended chapters are interconnected, but for each chapter stand-alone data will be generated. Therefore, unexpected delays in one of the approaches will not interfere with the completion of the final thesis. Year 1 and part of year 2 are dedicated to characterization of Heatin's effects on high temperature acclimation in *Arabidopsis*, to optimize Heatin bioactivity and to amend the molecule for biochemical purification of direct target proteins. Moreover, relevant bioinformatics courses will be followed. The optimally functioning Heatin-II compound will be used in Year 2 for transcriptome analysis and genome-wide association mapping, to detect and select candidate genes that are affected by the compound and contribute to HTAR. Moreover, biochemical purifications and LC-MS will be performed in year 2. For this I will travel and stay for ~2 a 3 months at Oxford University, UK. Year 3 and part of year 4 will be used to obtain functional, mechanistic and physiological insight in how these candidate genes control HTAR in *Arabidopsis* and relevant crop species/varieties. In year 4, manuscripts will be prepared and submitted to international peer-reviewed journals and presented at relevant (inter)national conferences. In addition, all datasets and generated plant lines will be deposited in public databases and repositories for the benefit of the plant sciences community. The second half of year 4 will be dedicated to writing the PhD dissertation and peer-reviewed scientific publications.

8. Economic and/or societal relevance

Describe the relevance of the results and/or insights from the research for and the contribution to solving societal and economic issues relevant to the topsector Horticulture and Starting Materials.

Humanity depends on products provided by plants for food, feed, biofuels, fibers, medicine etc. Presently, high temperature is already among the most damaging factors for plant productivity worldwide and current climate models predict that this will worsen, as global temperature will rise by 0.8-4^{o}C by the year 2100 [1,2,11,24,25]. Each degree Celsius increase leads to a three-to-ten percent decrease in crop yields. This is problematic since global food production needs to double to feed a growing world population. Although our major crops have been subjected to centuries of traditional trial-and-error breeding, only very few genetic markers for temperature effects on plant growth and acclimation are available. This is due to the fact that the variation in the germplasms is surprisingly limited [26]. The major thermosensory pathways remain to be discovered in plants and the lack of knowledge on such pathways hampers breeding efforts thermotolerance in crops. In this project the novel chemical compound Heatin, that stimulates high temperature acclimation, will be central. Aim is to identify Heatin's protein targets and affected genes/genetic networks to gain detailed mechanistic understanding of how high temperature-mediated growth acclimation is brought about in plants. I will first study the effect of Heatin on various phenotypes associated to high temperature acclimation in *Arabidopsis thaliana.* Next, Heatin's effect on relevant traits in crops will be assessed.
Since Heatin potentially affects whole gene families it is expected that this project will uncover novel thermosensory pathways that are presently unknown because their detection is hampered by genetic redundancy. Understanding how plants perceive temperature and translate the signal into relevant acclimation responses is of great relevance for the academic community as well as plant breeders who need to reduce the impact of high temperature on plant performance and productivity to maintain and even increase crop yield. Such knowledge is urgently needed in view of the enormous impact climate change will have on primary production. In addition, Heatin has great economic potential as it may directly be used as growth regulator. The potential of Heatin to prime crops to better withstand high ambient temperatures in the field or greenhouse is an appealing aspect of this proposal. I will study how this priming affects yield and quality/quantity of harvestable products. In addition, I will elucidate which crops/varieties are sensitive to Heatin-induced HTAR and if this depends on the same genetic mechanisms as in *Arabidopsis*. The results of this project therefore can be directly implemented in existing breeding programs to improve crop yield and quality of harvestable products under the regime of global warming. To facilitate this, all the generated datasets will be made publicly available through existing databases for the benefit of the whole plant research community and plant breeding industry and the results will be published in high ranking *peer-reviewed* scientific journals.

9. Composition of the consortium and in-kind matching

Indicate the private and/or public partners involved in the project and the in-kind matching that is required.

- Dr. Martijn van Zanten, Prof. Dr. Sjef Smeekens (Molecular Plant Physiology; Utrecht University)
- Dr. Stéphanie Robert (Swedish University of Agricultural Sciences, Umeå, Sweden)
- Dr. Martijn Fiers (Plant Research International, WUR)
- Prof. Dr. Roland J. Pieters, Dr. Nathaniel Martin (Department of Medicinal Chemistry & Chemical Biology, Utrecht Institute for Pharmaceutical Sciences, Utrecht University)
- Dr. Renier van der Hoorn (Plant Chemetics, Plant Science Department, Oxford University, UK)
- BEJO Zaden BV (Ir. Tjeerd Vrind, Dr. Bert Compaan, Dr. Corinne de Groot), Warmenhuizen

Daily supervisor of this project will be Dr. Martijn van Zanten. He joined the Molecular Plant Physiology group in 2012 with a personal NWO VENI fellowship to pursue his research interest in the mechanisms of high temperature stress acclimation. His previously held NWO Rubicon and EMBO (European Molecular Biology Organization) fellowships at the Max Planck Plant Institute for Plant Breeding and Genetics in Cologne, Germany, provided him with an excellent international position in this field that he wants to further build up with an own research group.

FINANCIAL DETAILS

10. Budget

Please use the table below for the description of the personnel and material resources required for the project. The maximum budget that can be applied for is k€ 250.

Project budget (k€)	Year 1	Year 2	Year 3	Year 4	Total
Personnel costs					
Salary PhD student	47	50	53	56.6	206.6
Research costs					
Consumables	15	18.5	5	4.9	43.4
Other (*in kind*)			7	11.8	18.8
Total	62	68.5	65	73.3	268.8

Specification research budget

All necessary equipment and standard growth facilities for successful execution of the proposed program are already present in the Molecular Plant Physiology laboratory or in the groups of Prof. Pieters (organic synthesis) and Dr. Van der Hoorn (pull down experiments and LC-MS).
In year 1 compounds need to be ordered for structure-activity studies. Some of these likely need to be resynthesized at the Chembridge company (5k). In addition, hormones and inhibitors need to be purchased (2k). Moreover, qRT-PCRs need to be performed (2.5k) and standard consumables are required (3k). Estimated costs for synthesis of Heatin-II and Heatin-II-Biotin are 2.5k. In year 2 consumables need to be purchased for pull down assays and LC-MS (5k). Moreover, microarray experiments will be performed (7.5k). Also travel and subsistence costs will be made for performing the pull down and LC-MS experiments at Oxford University, UK (3k) and standard consumables are required (3k). Year 3 and 4 requires consumables for molecular cloning and molecular physiological experiments (5k and 4.9k respectively). Bejo Zaden BV, Warmenhuizen, the Netherlands, intents to provide the relevant crop species/varieties and growth facilities as well as material and sequencing support for the identification of sequences of homologous of *Arabidopsis* genes responding to Heatin-II trough *in kind* contribution (see the letter-of-support that is that is attached to this proposal). These materials and facilities are required in Year 3 and 4 to meet the aims of Objective 5b.

CV PHD CANDIDATE AND PUBLICATIONS RESEARCH GROUP

11. Curriculum vitae proposed PhD candidate

11a. Personal details

Title(s), initial(s), first name, surname: BSc, L C, Lennard, van der Woude
Nationality: Dutch

11b. Bachelor study

University/College of Higher Education: Utrecht University
Name Bachelor study: Biology
Specialisation: Molecular and cellular plant biology / Developmental and chemical genetics.

Non-compulsory courses followed: Bio-ethics, Biotechnology, Developmental biology, Developmental biology and genetics II, Environmental change through time, Eukaryotic microbiology, Genome biology, History and philosophy of biology, Microscopy, Molecular genetics, Plants and climate change, Plant physiology, Theoretical ecology, Toxicology

Thesis title: Temperature-mediated developmental processes and thermosensors in plants
Thesis grade: 7.5
Date diploma: 29-07-2011
Average grade: 6.88

11c. Master study

University: Utrecht University
Name Master study: Molecular and Cellular Life Sciences
Subjects year 1 (*List the subjects and courses followed*):

- Introduction course (1 ECTS)
- Systems biology (3 ECTS)
- Major research project (51 ECTS)
- 10 Seminars (1.5 ECTS)

Average grade year 1: 8.1
Specialisation (year 2; *Mention your chosen specialisation and list the subjects and courses followed*):
Specialisation: Molecular plant physiology / Chemical genetics
Courses:

- Minor Research project (33 ECTS)
- Electives: Following up on minor research project (12 ECTS)
- Intracellular membrane processes (3 ECTS)
- Biotechnology (5 ECTS, to complete)
- Molecular recognition (3 ECTS, to complete)

Average grade year 2 (*If year 2 has not been completed yet, please provide the average grade so far*): NA
Graduation date: (*Date can be in the future*): Feb 2014
Title thesis: (*Proposed or completed thesis, please indicate status*): Unraveling the molecular mechanism underlying heat acclimation in plants using the novel growth regulator Heatin (This proposal counts as my Master thesis).
Thesis grade: NA

11d. Other academic activities

Please provide information about (extra)curricular academic activities the candidate has engaged in, for example, membership of committees or the involvement in the organisation of conferences (max 200 words).
I attended the annual Netherlands Organization for Scientific Research (NWO, ALW) annual Experimental Plant Science meeting in Lunteren in 2012, the Umeå Renewable Energy Meeting 2013 at Umeå University in Umeå, Sweden and the Auxin Sailing 2013 meeting: 2nd International Meeting on Early Auxin Research in Leiden, June 2013.

11e. Scholarships and prizes (if applicable)

- Erasmus Placement Scholarship for Minor Research Project in Sweden
- Facility access and support grant for our chemical genomics project; Laboratories for chemical biology, Umeå, Sweden (By; Van der Woude L, Robert S, Franklin K, Proveniers MCG, Van Zanten M)

11f. Scientific output of proposed candidate (if applicable)

If available, please mention below a maximum of 5 scientific publications or other relevant scientific output of the proposed candidate.
I wrote a facility access and support grant for our chemical genomics project; Laboratories for chemical biology, Umeå, Sweden (By; Van der Woude L, Robert S, Franklin K, Proveniers MCG, Van Zanten M).

11g. Candidate's motivation

Please provide your motivation for a PhD position (max 200 words)
I am currently following the master's program 'Molecular Cellular Life Sciences' at Utrecht University. I am in the second year of this program. As part of the program I have completed a 9 month internship at the Molecular Plant Physiology group at Utrecht University followed by a 6 month internship at the Umeå Plant Science Centre at the Swedish Agricultural University in Umeå, Sweden. During these internships I got acquainted with and subsequently charmed by experimental plant sciences. I learned that it is a fascinating and challenging field of biology.
Next to its fascinating biological background, plant science is of great importance to our global society on a multitude of issues. It is of particular relevance to the Dutch economy through plant breeding companies and its large agricultural sector. With this knowledge in mind, I became determined to continue my education in the field of experimental botany

after finishing my master's education. I have a strong wish to follow a PhD-training in the field of molecular plant genetics as this will prepare me optimally for a position in academia or at a plant breeding company.

11h. Title(s), initial(s), surname and university of the two references expressing support for the application on the two recommendation letters accompanying the application

Name: Drs. Anje de Graaf
University: Utrecht University (Coordinator master programme; Molecular and Cellular Life Sciences)

Name: Dr. Stéphanie Robert
University: Swedish Agricultural University, Umeå, Sweden (Internship supervisor)

12. Top 5 publications of the research group related to the proposed research

1. Bours R, Van Zanten M, Pierik R, Bouwmeester H, Van der Krol A (2013) Light and temperature cycles affect PHYB-controlled ethylene signalling; Limiting leaf movement and growth of *Arabidopsis*. ***Plant Physiology*** 163: 882-895 **(IF: 6.5)**

2. Proveniers M, Van Zanten M (2013) High temperature acclimation through PIF4 signaling. ***Trends in Plant Science*** 18: 59-64 **(IF: 11.0)**

3. Van Zanten M, Voesenek LACJ, Peeters AJM, Millenaar FF (2009) Hormone- and light-mediated regulation of heat-induced differential petiole growth in *Arabidopsis thaliana.* ***Plant Physiology*** 151: 1446-1458 **(IF: 6.5)**

4. Li P, Wind JJ, Shia X, Zhanga H, Hanson J, Smeekens SC, Teng S (2011) Fructose sensitivity is suppressed in *Arabidopsis* by the transcription factor ANAC089 lacking the membrane bound domain. ***Proceedings of the National Academy of Sciences USA*** 108: 3436-3441 **(IF: 9.7)**

5. Tessadori F*, Van Zanten M*, Pavlova P, Clifton R, Pontvianne F, Snoek LB, Millenaar FF, Schulkes R-K, Van Driel R, Voesenek LACJ, Spillane C, Pikaard CS, Fransz P, Peeters AJM (2009) PHYTOCHROME B and HISTONE DEACETYLASE 6 control light-induced chromatin compaction in *Arabidopsis thaliana*. ***PLoS Genetics*** 5: e1000638 *equal contribution **(IF: 8.7)**

STATEMENTS BY THE APPLICANT

13. Statements by the applicant

YES I endorse and follow the Code Openness Animal Experiments (if applicable)

YES I endorse and follow the Code Biosecurity (if applicable)

YES I have completed this form truthfully

Name: Prof. J.C.M. Smeekens
Place: Utrecht
Date: 10-10-2013

Please submit the application to NWO in electronic form (pdf format is required!) using the Iris system, which can be accessed via the NWO website (www.iris.nwo.nl). The application must be submitted from the account of the main applicant. For any technical questions regarding submission, please contact the IRIS helpdesk (iris@nwo.nl).