Improving cold tolerance in tomato plants using natural variation

Master Thesis by MSc student Bart André, student number: 3568008
Date: 2012-06-12

The maximum length of a proposal is 11 pages.

1a. Details of proposal

Title: Improving cold tolerance in tomato plants using natural variation.

Area: O Geo and Biosphere • from Molecule to Organism

Summary (scientific summary in English, max. 250 words):

The demand for food and fuel is increasing due to the rising human population. Therefore are their new strategies needed to increase crop yield. Plants suffer from low temperatures which leads to damage to fruit and plant growth. Even non freezing temperatures can have a dramatic effect on plant development. Better insight into cold acclimation responses of plants will lead to better protection to low temperatures. One well examined process that is active during cold acclimation is the c-repeat-binding factor (CBF) regulon. This CBF regulon regulates multiple genes that in turn are responsible for a cold acclimation response. However, not all reactions of the plant are explained by the CBF regulon, therefore more pathways need to be involved.
This proposal will search for other pathways that are involved in cold acclimation. This will be done by analyzing tomato varieties that grow on high altitudes with 1) micro array studies and 2) QTL mapping. Identified candidate genes will be studied in function by transforming Arabidopsis plants. It will also be tested if these candidate genes are connected to the CBF regulon or if they regulated the CBF regulon. Heretofore CBF knockout mutants of Arabidopsis will be used and candidate genes will be introduced. The newly identified related genes can be used to improve crop performance even further and keep up with human demand.

1b. Details of applicant

Name: Bart André

Gender: • Male O Female

Date of birth: 22-03-1988

Institution: Utrecht University

Position: O Professor O Associate professor (UHD) O Assistant professor (UD) • Other: Master student

Permanent position: O Yes • No, end date contract: n/a

E-mail: b.j.andre@students.uu.nl

Research School: Utrecht University

Name and address of the responsible person at your institution (e.g. scientific director of the institute or dean of the faculty): Prof.dr. Han Wösten, Padualaan 8, 3584CH, Utrecht

1c. Alternative contact

Name: dr. R. Pierik (Ronald)
Tel: 030 2536838
Email: r.pierik@uu.nl

1d. Renewed application? O Yes • No

In case of a renewed application please indicate the file number of the previous application and summarize the main changes

1e. Applying for: • PhD student O Post Doc O Ship time

1f. Composition of the research group
List all staff members involved in the proposed research: provide name, initials, titles and type of involvement, e.g. daily guidance, technician, thesis supervisor, advisor.

Name and title	Specialization	Institution	Involvement
prof. dr. L.A.C.J. Voesenek	Head of Group	Utrecht University	Advisor
dr. R. Pierik	Principal Investigator	Utrecht University	thesis supervisor, daily supervisor
dhr. ing. R.A.M. Welschen	Technician	Utrecht University	technician

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

Gewassen ondervinden schade door lage temperaturen en vorst waardoor de groei van de plant achterblijft of zelfs delen afsterven. Omdat landbouwgrond schaars is (en schaarser zal worden) is het belangrijk om gewassen te ontwikkelen die beter groeien in gebieden met lage temperaturen. Een van de processen die een rol spelen bij bescherming tegen lage temperaturen is het moleculaire CBF regulon. In dit onderzoek zal onderzocht worden hoe tomaten planten verbeterd kunnen worden in de regulatie van processen die beschermen tegen koude temperaturen waardoor ze minder schade leiden. Tevens zal er gebruik gemaakt worden van natuurlijke variatie die voorkomt in tomaten planten om zo de planten te verbeteren.

3. Description of the proposed research
Max. 4 pages (and max. 3600 words), including figures, excluding literature references). Include details of objectives, innovative aspects, scientific approach, impact, and literature references

Low temperatures are a problem in agriculture because they negatively affect plant growth and development. Better regulation of cold acclimation (CA), which leads to acquiring chilling or freezing tolerance, will make low temperatures a less severely limiting factor for plant growth and development (Theocharis *et al.*, 2012; Fanucchi *et al.*, 2012). To illustrate the problem: throughout 15 December 2010 Florida was hit with consecutive nights of freezing weather. This causes damage to winter tomato production in the Immokalee area up to 70-80% of the crop (see www.thepacker.com)

Freezing causes mainly damage to the membrane systems of cells, which happens through severe dehydration. When temperatures drop below 0°C ice will form in extracellular and intercellular spaces. Because the chemical potential of ice is lower than liquid water, the water potential outside the cell will drop below of that inside the cell when extracellular ice is forming. Now unfrozen water will flow through water potential gradient from inside the cell to intercellular spaces. This damage caused by frost-induced dehydration to membranes is the main problem of frost temperatures (Thomashow, 1999). Therefore changes during CA implicate stabilization of essential membranes,

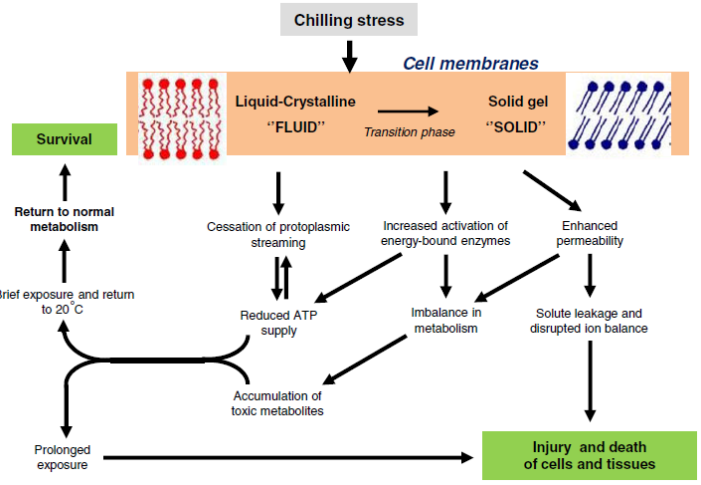


Figure 1. Model that represents processes that are connected to a cold acclimation response. The damage of membranes is the primary signal for cold stress and results in a cascade of processes that affect the plant (Theocharis *et al.*, 2012).

although CA involves more than membrane stabilization alone. In response to lower, but non-freezing temperatures, plants display a range of biological changes to prepare for winter and to increase freezing tolerance (see figure 1). Biological modifications that are involved in cold acclimation include reduced photosynthesis, changes in sugar content, cellular transport and cell membrane, production of anti-freezing proteins, imbalanced metabolism, and lower respiration (Sandve *et al.*, 2011). These processes are differently regulated in specific tissue, like leaves and crown tissue. A short, 1 or 2 days period of lower temperature induces cold-protective proteins that are rapidly removed by increasing temperatures (Medina *et al.*, 2011).

Different signals, like light and temperature, are indicators for plants for a seasonal change. Cereal species and also other crops have developed different strategies to deal with winter; there are winter-hardy and winter-sensitive varieties (Carvallo *et al.*, 2011). Both varieties need a period of low temperature to adjust to winter. Winter-hardy cereal varieties also need a period of cold before they flower, which is called vernalization. Cold acclimation and vernalization are processes that are regulated upon lowering temperatures, and are most likely connected (Winfield *et al.*, 2010).
The regulation of cold acclimation is largely unknown, but many genes are differentially regulated upon cold treatment. It is possible that some genes are induced by a state of dehydration and some by cold during cold acclimation. Although most research on CA is done with the genetic plant model *Arabidopsis thaliana* the function of most genes that are cold regulated is unknown. The standing hypothesis so far is that temperature-mediated alterations in membrane fluidity/rigidity are the primary signal for CA (Winfield *et al.*, 2010).

One well examined mechanism that leads to cold acclimation is regulated by c-repeat-binding factors (CBFs)/dehydration-responsive element-binding factors (DREs) (Liu *et al.*, 1998). These CBFs/DREs bind to corresponding c-repeat/dehydration response elements that are present in many promoters of already identified cold responsive genes. These transcription factors and their target genes are collectively referred to as the CBF regulon (see figure 2, Medina *et al.*, 2011). This regulon is extensively examined in *Arabidopsis* and is known as the CBF cold response pathway. Plants exposed to low temperature rapidly change gene expression, resulting in alterations of transcript levels of hundreds of genes (Zhu *et al.*, 2007). The first genes that are induced upon low temperatures are *CBF1*, *CBF2* and *CBF3*, also known as *DREB1b*, *DREB1c* and *DREB1a*.

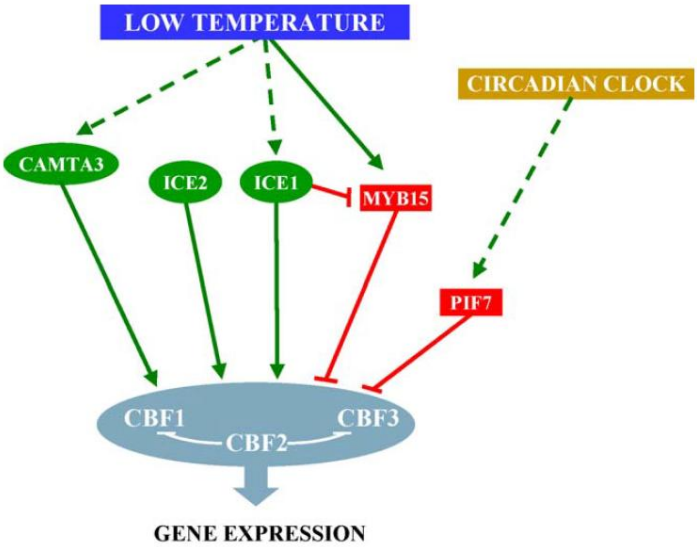


Figure 2. Trans-acting regulation of CBF expression. ICE1, ICE2 and CAMTA3 positively regulate CBF expression. MYB15 and PIF7 act as negative regulators. The negative regulation of CBF1 and CBF3 by CBF2, and of MYB15 by ICE1 is also represented. Broken arrow means activation by unknown mechanisms (Medina *et al.*, 2011).

The *CBF2* promoter contains ICEr1 and ICEr2 (for Inducer of CBF expression region 1 and 2) elements that are sufficient for cold induced expression (Zarka *et al.*, 2003). ICE1 is a transcription factor that is constitutively expressed, however only under low temperatures can ICE1 activate *CBFs*. Speculation is that ICE1 protein needs cold-induced modification or a transcriptional cofactor to activate the expression of *CBFs*. This ICE1 is a myelocytomatosis oncogene (MYC) basic helix-loop-helix (bHLH) transcription factor that binds to MYC elements in CBF promoters (Lee *et al.*, 2005). The Arabidopsis mutant *ice1* shows enhanced expression of *CBF2* after 6 and 12h of cold treatment

(Chinnusamy *et al.*, 2003). This supported the idea that CBF factors are mediated by ICE1-like proteins. Recently ICE2, also a bHLH transcription factors, was identified regulating *CBF1* (Fursova *et al.*, 2009). CAMTA3 (from the calmodulin binding transcriptional activators family) is also a transcription factor involved in positive regulation of CBF upon low temperatures (Doherty *et al.*, 2009).

Two negative regulators of CBFs are known, MYB15 and PIF7, MYB15 binds to MYB promoter elements of *CBF1*, *CBF2* and *CBF3*. MYB15 knockout Arabidopsis mutants are better in activation of cold acclimation confirming their negative role. Also is MYB15 involved with ICE1 interactions, whereby ICE1 negatively regulates MYB15 (Agarwal *et al.*, 2006). However this CBF cold response pathway cannot explain all regulatory changes made upon cold stress. Probably more and different mechanisms are involved located on different loci (Medina *et al.*, 2011).

The CBF cold response pathway is found in freezing-tolerant (FT) and in freezing-sensitive (FS) plants, like *Brassica napus*, barley (both FT) (Skinner *et al.*, 2005), tomato and rice (both FS) (Zhang *et al.*, 2004). The CBF regulon of tomato and rice, both FS, consists of +/- 10 inducible cold responsive genes. However, in Arabidopsis and poplar, both FT, the CBF regulon consists of +/- 85 and 63 cold inducible genes. This difference in gene expression is most likely responsible for different cold acclimation responses of plants (Carvallo *et al.*, 2011). Zhang et al. (2004) identified three CBF homologues in *Lycopersicon esculentum* (tomato, also known as *Solanum lycopersicum*) and tested them in response to cold acclimation. These genes were located on tomato chromosome 3 between molecular markers TG520 and CT141 and are named *LeCBF1*, 2 and 3. Analysis showed that only *LeCBF1* is activated upon low temperatures and probably responsible for a cold acclimation response (Zhang *et al.*, 2004). Also other tomato homologues of cold responsive genes were identified and their expression was measured. However these genes were not affected by CBF expression. This made the authors hypothesize that the tomato CBF regulon is significantly smaller and has a limited function compared to the Arabidopsis CBF regulon.

This research proposal will use the knowledge of the CBF regulon to explore possibilities to improve cold acclimation in tomato plants, through a combination of research strategies. Furthermore, I propose to search for alternative mechanisms involved by exploring existing natural variation in tomato.

Scientific approach

Searching for alternative pathways in tomato for regulation of cold acclimation

To identify new and important key players in cold acclimation response other accessions of tomato plants will be explored. Tomato plants (seeds will be ordered) from high altitude (thus adapted to lower temperatures, figure 3) will be used to pinpoint quantitative trait loci (QTL) or gene(s) for further research and possible inbreeding in commercial available tomato plants. This will be done by two approaches, 1) using transcriptomics; analyzing available datasets of tomato cDNA microarrays, run micro arrays through the current proposal, selecting possible candidate genes and studying their expression in the high altitude varieties by PCR and 2) via QTL mapping of high altitude varieties crossed with commercial tomato plants. Results of these approaches will be combined to get more insight into CA.

First the high altitude plants need to be tested for their degree in CA. Variations that will be used are *S. chilense*, *S. chmielewskii*, and *S neorickii* which will be compared to *S. lycopersicum* (see figure 3, Nakazato *et al.*, 2010). To test the ability of these plants acclimate to low temperatures they will be treated as following: first grown at 20°C for 4 weeks, then a slow decline in temperature to 13°C in one week, followed by a rapid cold shock of 4°C for seven days. Some plants will return to 20°C and some will be exposed to chilling temperatures of -4°C for seven days. This experimental design will be used to give plants a chance to activate a CA response, which is the most realistic mimic of natural cold conditions. After a week the damage to the plants will be examined by scoring phenotypic characteristics: plastochron index, rate of seed germination and growth, chlorophyll fluorescence and shoot wilting. From these plants used for checking CA, leaf and crown samples will

be taken for mRNA expression profiling. These samples will be taken before the plants are transferred to the previously described temperature condition. New plants will be used for collecting the samples at the same conditions for microarray-based transcriptome profiling (GeneChip Tomato Genome Array from Affymetrix). Three time points (week 4 at 20°C, week 6 at 4°C, week 7 at -4°C) will be taken and expression of mRNA in leaves and crown tissue will be measured. The reason for taking samples from these organs is because cold-responsive genes are differently expressed between different tissues (Ganeshan *et al.*, 2008). It is also shown that plant survival is depended of specific tissue within the crown (Livingston *et al.*, 2006). While waiting for the data of our own micro array, available datasets of tomato cDNA microarrays will be analyzed for identifying possible candidate genes that are regulated during CA. This data and our own data will be analyzed by bio-informatics.

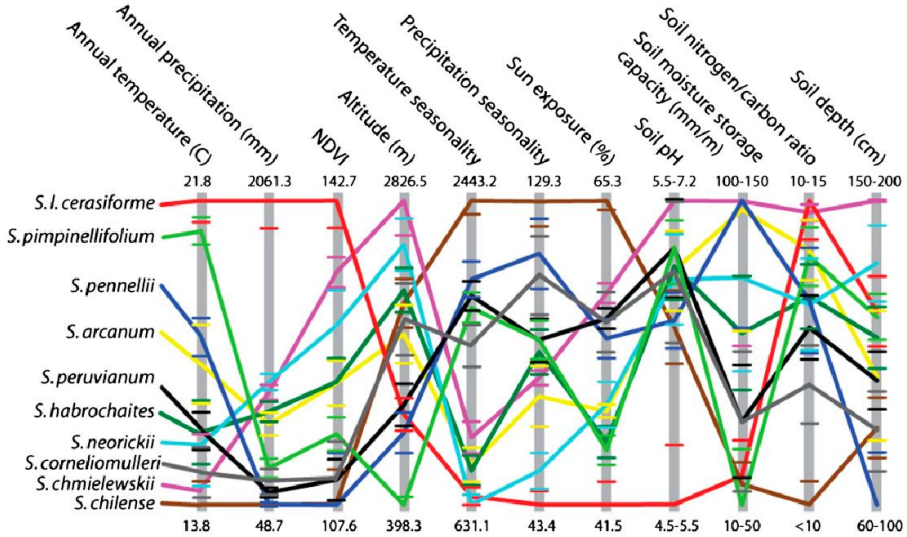


Figure 3. A graph showing environmental variation of tomato species (Solanum). The different colors represent the different tomato species. The vertical bars represent the different environmental variabilities that were studied, like annual temperature and altitude (Nakazato *et al.*, 2010).

For making a linkage mapping many populations need to be made and this will take a long time since no recombinant inbred lines are available. Therefore this work will start at the beginning of the project even before knowing which accession has the best cold acclimation response. of *S. lycopersicum* x *S. chilense*, *S. lycopersicum* x *S. chmielewskii* and *S. lycopersicum* x *S. neorickii* will be made. These will be backcrossed and generations of intermating and generations of selfing (1 generation 3-4 months, 5-6 generations) will be made. Using the genome sequence of tomato that is available via Sol Genomics Network or via TIGR Tomato Gene Index candidate genes will be identified from the QTLs. If a QTL contains many genes, more research needs to be done to limit the possible candidate genes. This will be done by looking further into gene function, connection to other genes, protein function (prediction), promoter domains and other molecular functions.

Testing the functionality of candidate genes

When one or more candidate gene(s) are found they will be further analyzed. This will be done by over expression (35S promoter constructs) of the tomato genes in *Agrobacterium tumefaciens* mediated transformed Arabidopsis plants to check for a CA phenotype. This will give a conformation that the gene is involved in CA.

Testing these candidate gene(s) will lead to a more complete picture of the cold acclimation response. This knowledge can be used to search for other mechanisms in Arabidopsis or tomato that may be connected to CBF regulon. For analyzing if the candidate gene(s) is regulated by the CBF regulon the promoter will be searched for CRT/DRE elements and AP2/ERF-binding domains, which are common elements in other CBF regulated genes. Another approach will be to test the candidate gene(s) in functionality to CA in an Arabidopsis CBF knockout mutant. With this approach even new factors in CA can be found when regulated by these candidate gene(s). These interactions with possible other genes can than be further studied in future plans.

Overall, these experiments will lead to a better understanding of CA in plants, especially in tomatoes, and give an insight in other pathways that are connected to the CBF regulon. This will provide breeding companies with new related target genes for improving their crops in CA. This new information that will be found can also be used to look to other important crop species in order to improve cold acclimation.

Bottleneck

A problem that can occur by creating cold-resistant plants is that they acquire the need for vernalization. First we need to discover if the high altitude tomato varieties have vernalization needs. If not the chance is limited that we create it in the crossings. This means that we have to check if the tomato varieties flower without induction of low temperatures. If these tomato varieties have vernalization needs then this could be a potential problem for growers. To address this problem we have to, in future plans, look closer at the process of vernalization and genes that are involved so this trait is not transferred.

References

Agarwal M, Hao Y, Kapoor A, Dong CH, Fujii H, Zheng X, Zhu JK (2006) A R2R3 type MYB transcription factor is involved in the cold regulation of CBF genes and in acquired freezing tolerance. J Biol Chem 281(49)

Carvallo MA, Pino MT, Jeknic Z, Zou C, Doherty CJ, Shiu SH, Chen TH, Thomashow MF (2011) A comparison of the low temperature transcriptomes and CBF regulons of three plant species that differ in freezing tolerance: Solanum commersonii, Solanum tuberosum, and Arabidopsis thaliana. J Exp Bot 62(11)

Chinnusamy V, Ohta M, Kanrar S, Lee BH, Hong X, Agarwal M, Zhu JK (2003) ICE1: a regulator of cold-induced transcriptome and freezing tolerance in Arabidopsis. Genes Dev 17(8)

Doherty CJ, Van Buskirk HA, Myers SJ, Thomashow MF (2009) Roles for Arabidopsis CAMTA transcription factors in cold-regulated gene expression and freezing tolerance. Plant Cell 21(3)

Fanucchi F, Alpi E, Olivieri S, Cannistraci CV, Bachi A, Alpi A, Alessio M (2012) Acclimation increases freezing stress response of Arabidopsis thaliana at proteome level. Biochim Biophys Acta 1824(6)

Fursova OV, Pogorelko GV, Tarasov VA (2009) Identification of ICE2, a gene involved in cold acclimation which determines freezing tolerance in Arabidopsis thaliana. Gene 429(1-2)

Ganeshan S, Vitamvas P, Fowler DB, Chibbar RN (2008) Quantitative expression analysis of selected COR genes reveals their differential expression in leaf and crown tissues of wheat (Triticum aestivum L.) during an extended low temperature acclimation regimen. J Exp Bot 59(9)

Lee BH, Henderson DA, Zhu JK (2005) The Arabidopsis cold-responsive transcriptome and its regulation by ICE1. Plant Cell 17(11)

Liu Q, Kasuga M, Sakuma Y, Abe H, Miura S, Yamaguchi-Shinozaki K, Shinozaki K (1998) Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in Arabidopsis. Plant Cell 10(8)

Livingston DP, Premakumar R, Tallury SP (2006) Carbohydrate partitioning between upper and lower regions of the crown in oat and rye during cold acclimation and freezing. Cryobiology 52(2)

Medina J, Catala R, Salinas J (2011) The CBFs: three arabidopsis transcription factors to cold acclimate. Plant Sci 180(1)

Nakazato T, Warren DL, Moyle LC (2010) Ecological and geographic modes of species divergence in wild tomatoes. Am J Bot 97(4)

Sandve SR, Kosmala A, Rudi H, Fjellheim S, Rapacz M, Yamada T, Rognli OA (2011) Molecular mechanisms underlying frost tolerance in perennial grasses adapted to cold climates. Plant Sci 180(1)

Skinner JS, von Zitzewitz J, Szucs P, Marquez-Cedillo L, Filichkin T, Amundsen K, Stockinger EJ, Thomashow MF, Chen TH, Hayes PM (2005) Structural, functional, and phylogenetic characterization of a large CBF gene family in barley. Plant Mol Biol 59(4)

Theocharis A, Clement C, Barka EA (2012) Physiological and molecular changes in plants grown at low temperatures. Planta

Thomashow MF (1999) PLANT COLD ACCLIMATION: Freezing Tolerance Genes and Regulatory Mechanisms. Annu Rev Plant Physiol Plant Mol Biol 50

Winfield MO, Lu C, Wilson ID, Coghill JA, Edwards KJ (2010) Plant responses to cold: Transcriptome analysis of wheat. Plant Biotechnol J 8(7)

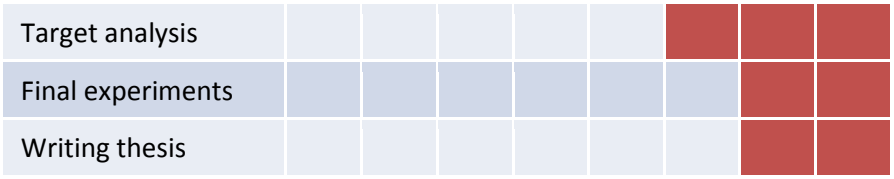
Zarka DG, Vogel JT, Cook D, Thomashow MF (2003) Cold induction of Arabidopsis CBF genes involves multiple ICE (inducer of CBF expression) promoter elements and a cold-regulatory circuit that is desensitized by low temperature. Plant Physiol 133(2)

Zhang X, Fowler SG, Cheng H, Lou Y, Rhee SY, Stockinger EJ, Thomashow MF (2004) Freezing-sensitive tomato has a functional CBF cold response pathway, but a CBF regulon that differs from that of freezing-tolerant Arabidopsis. Plant J 39(6)

Zhu J, Dong CH, Zhu JK (2007) Interplay between cold-responsive gene regulation, metabolism and RNA processing during plant cold acclimation. Curr Opin Plant Biol 10(3)

5. Timetable of the project

	Year 1		Year 2		Year 3		Year 4	
Initial experiment								
RIL populations								
Microarray								
Analyses microarray								
QTL mapping								



6. Societal significance

The ability to improve CA, through detailed knowledge generated in this proposal will help increase tomato yield. Firstly, it can extend the growing season since also at lower temperatures CA tomatoes could still be grown. Secondly, areas which were previously not suitable due to unfavorable temperatures may become useful for growth of tomato genotypes with enhanced cold tolerance. Furthermore, it may help reduce energy expense in greenhouses because of lower growing temperature. Finally, genotypes with increased CA may suffer less damage to plant and fruit from chilling temperatures.

7. Budget

		Year 1	Year 2	Year 3	Year 4
Personnel (mm)		41	49	51	59
Research costs (k€)					
	Equipment	4	6	6	3
	Consumables*	13	7	4	3
	Fieldwork/Travel*	1	1	1	1

* The sums requested for consumables and fieldwork/travel expenses combined should not exceed 50,000 euro for the entire grant period.

Specification of the requested funds:
Equipment: microarray analysis software, QTL mapping software
Consumables: microarray chips, general molecular biology kits
Fieldwork/Travel: congresses

8. Statements by the applicant

YES/~~NO~~ I endorse and follow the Code Openness Animal Experiments (if applicable)

YES/~~NO~~ I endorse and follow the Code Biosecurity (if applicable)

YES/~~NO~~ I have completed this form truthfully

Name: Bart André
Place: Utrecht
Date: 25-05-2012

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).