# Cellular stress responses during cryo-induced stress in *Arabidopsis* shoot tips

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## PUBLICATIONS AND MANUSCRIPTS RELATED TO THE SUBMITTED THESIS

Some parts of the following study resulted in accepted paper (1), or submitted manuscripts (2).

1. **Accepted Paper:**

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**Stock, J.,** Rutten, T., Schwarzländer, M., Keller, E.R.J. and Mock, H.-P. (2018). Cell wounding induced *PR5* expression in *Arabidopsis* shoot tips under the control of the transcription factor *WRKY22*.

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## ABBREVIATIONS

| | |
|---|---|
| aa | amino acids |
| ABA | abscisic acid |
| acc. | acclimated |
| bp | base pairs |
| CaMV | cauliflower mosaic virus |
| CLSM | Confocal Laser Scanning Microscopy |
| $CO_2$ | carbon dioxide |
| DEG | differentially expressed gene |
| DMSO | dimethyl sulfoxide |
| DNA | deoxyribonucleic acid |
| DSC | differential scanning calorimetry |
| DTT | dithiothreitol |
| EDTA | ethylenediamineteraacetic acid |
| FC | fold change |
| flg22 | flagellin |
| for | forward |
| FW | fresh weight |
| $GA_3$ | gibberellic acid |
| GFP | green fluorescent protein |
| GO | gene ontology |
| $H_2O_2$ | hydrogen peroxide |
| IAA | indole-3-acetic acid |
| JA | jasmonic acid |
| KO | knockout |
| LN | liquid nitrogen |
| MDA | malondialdehyde |
| min | minute |
| n. acc. | non acclimated |
| PCA | principal component analysis |
| PEG | polyethylene glycol |
| PR | pathogenesis-related |
| PVS | plant vitrification solution |
| qRT-PCR | quantitative real-time polymerase chain reaction |
| rev | reverse |
| RNA | ribonucleic acid |
| *RNA-seq* | RNA sequencing |
| roGFP | reduction-oxidation sensitive green fluorescent protein |
| ROS | reactive oxygen species |
| RT | room temperature |
| SA | salicylic acid |
| SAM | shoot apical meristem |
| SD | standard deviation |
| TEM | transmission electron microscopy |
| TPM | transcript per million reads |
| TF | transcription factor |
| WT | wildtype |
| zn | zink finger |
| x *g* | times gravity |

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## I SUMMARY

Cryopreservation, the long-term storage at ultralow temperatures at -196 °C, is a frequently used method in the context of conserving live plant tissue. *Ex situ* plant gene banks use cryopreservation to maintain genetic resources from vegetatively-reproduced plants or to preserve selected clones of highly heterozygous species.

However, the molecular basis underlying cryopreservation remains far from understood. Therefore, the shoot tips of the model plant *Arabidopsis thaliana* were subjected to a PVS2 Droplet Vitrification protocol. The established method ensured high recovery and was verified with a collection of *Arabidopsis* wildtype genotypes.

Two candidate genes *PATHOGENESIS RELATED GENE 5* (*PR5*) and the transcription factor *WRKY22* were characterized towards their impact during cryopreservation.

A transcriptomic approach, comparing wildtype (WT) and *wrky22* knockout (KO) shoot tips, unraveled that cryoprotectant treatment induced the degradation of meristematic cells as a result of changes in RNA processing and primary metabolism. A number of putative downstream targets of *WRKY22* were identified, related to phytohormone-mediated defense, to the osmotic stress response or to developmental processes. There were also alterations in the abundance of transcript produced by genes encoding photosynthesis-related proteins. The *wrky22* mutant plants showed an open stomata phenotype in response to their exposure to cryoprotectant treatment. *WRKY22* likely regulates a transcriptional network during the cryopreservation procedure, linking the explant's defense and osmotic stress responses to changes in its primary metabolism.

*PR5* transcript expression was enhanced after shoot tip preparation and during osmotic stress, dependent on the strength of tissue injury and the accompanied hydrogen peroxide ($H_2O_2$) burst. *PR5* expression was dependent on the presence of the functional *WRKY22* transcript. Reduced recovery of double knockout mutants suggested that *PR5* expression is regulated by *WRKY22*.

*Arabidopsis* represents a suitable platform for identifying the mechanistic basis of the response to the multiple stresses imposed by the cryopreservation process. The conclusions reached from the present analysis should be relevant for some important crop species, notably potato, for which high post-cryogenic viability is still limited to certain germplasm accessions.

## II ZUSAMMENFASSUNG

Kryokonservierung dient der Langzeitlagerung von pflanzlichen Geweben in flüssigen Stickstoff (-196 °C). Speziell in *ex situ* Genbanken wird Kryokonservierung für den Erhalt pflanzengenetischer Ressourcen genutzt, die nicht über Saatgut aufbewahrt oder vegetativ vermehrt werden.

Die biologisch relevanten Mechanismen während der Kryokonservierung sind weitgehend unerforscht. Daher wurden Sprossspitzen der Modelpflanze *Arabidopsis thaliana* mit einem PVS2 Tröpfchen Vitrifikationsprotokoll behandelt, was bei einer Vielzahl von Wildtypen (WT) hohe Regenerationsraten sicherte.

Die Kandidatengene *PATHOGENESIS RELATED GENE 5* (*PR5*) und der Transkriptionsfaktor *WRKY22* wurden molekular charakterisiert, um ihren Einfluss auf den Erfolg der Kryokonservierung zu verstehen.

Zunächst wurde eine Transkriptomstudie mit *Arabidopsis* WT und *wrky22* knockout (KO) Sprossspitzen durchgeführt. Die Ergebnisse zeigten die Auswirkungen der Behandlung mit Kryoprotektoren auf den Abbau meristematischer Zellorganellen mittels induzierter RNA Prozessierung und Veränderungen im Primärmetabolismus. Es wurden putative Interaktionspartner von *WRKY22*, die in Beziehung mit der Phytohormon-abhängigen Verteidigung, osmotischer Stressantwort und Entwicklungsprozessen stehen, identifiziert. Weiterhin beeinflusste *WRKY22* die Photosynthese und die KO Mutante wies nach Kryoprotektorbehandlung einen „*open stomata“* Phänotyp auf. Während der Kryokonservierung reguliert *WRKY22* ein Transkript-Netzwerk wodurch Verteidigungsmechanismen mit der osmotischen Stressantwort verlinkt werden und letztendlich den Primärmetabolimus beeinflussen.

Die Transkript-Expression von *PR5* wurde durch die Sprossspitzenpräparation und osmotischen Stress, abhängig von der Stärke der Gewebsbeschädigung und der damit einhergehende Wasserstoffperoxid ($H_2O_2$) Akkumulation, induziert. Die von *WRKY22* abhängige *PR5* Expression und die verminderte Regenerationsfähigkeit von Doppelmutanten deuteten darauf hin, dass *PR5* unter der Kontrolle von *WRKY22* steht.

Sprossspitzen von *Arabidopsis* erwiesen sich als geeignetes Modelobjekt um grundlegende Kryostress-induzierte Mechanismen zu verstehen. Die neuen Erkenntnisse sollen zukünftig auf Nutzpflanzen wie Kartoffeln angewendet werden, um die Regeneration nach der Kryokonservierung zu verbessern.

# CHAPTER 1. INTRODUCTION

## 1.1. Major threats of plant diversity

Plant biodiversity disappears rapidly as a direct impact of humankind uses of plant natural resources. As a consequence, 25 % of the in total estimated 400,000 plant species worldwide are currently endangered. The major threats to species at risk of extinction are habitat loss and degradation because of urban development or extensive agricultural use. Natural biotope destruction caused by human activity is the primary risk for 83 % of endangered plant species. Humans encourage invasion of species that are found out of its normal range and therefore called alien species. They compete or even hybridize with native plants and alter abiotic environment. Unsustainability use of plant population, as well as the environmental pollution by chemical, biological or radioactive contaminants reduces plant production and whole ecosystem may be vulnerable to other threats (http://www.bgci.org/plant-conservation). It is of tremendous importance to preserve genetic resources which are otherwise irretrievably lost for classical and modern genetic engineering, plant breeding programs, agricultural uses, as well as a source of pharmaceutical compounds, and food industries.

## 1.2. Approaches to plant conservation

### 1.2.1. Sustainability and conservation strategies

Most of our ecosystems have been used unsustainably. The serious degradation needs to be reversed through general changes in policy and practice. In addition, *in situ* and *ex situ* conservation are two principle approaches to conserve plant germplasm and were designed to reinforce and complement each other.

*In situ* conservation includes preservation of ecosystems and the maintenance of viable populations in their natural habitats. Since natural biotopes have to cope with several threats, protected areas need to be developed and managed. *Ex situ* conservation means preservation and propagation of plants outside their natural surroundings. Species are removed from their natural settings and placed within the care of humans, like in botanical gardens or gene banks. Therefore, *ex situ* plant conservation provides crop diversity for long-term usage and protect germplasm from environmental hazards, diseases and human damage.

### 1.2.2. Strategies for *ex situ* germplasm conservation

Since the 1970s, large numbers of landraces and wild relatives of cultivated crops have been stored in *ex situ* plant gene banks. Gene banks established several plant conservation and propagation techniques, starting from seed storage, in field propagation, tissue culture or cryopreservation (Engelmann and Takagi, 2000) (**Figure 1**). For any given genepool, different complementary approaches are useful for efficient, safe, and cost-effective conservation. The strategy depends mainly on biological characteristics of the plant material, human use, the given infrastructure for conservation, the availability of germplasm, as well as political and administrative aspects (Withers, 1993). For instance, the Gatersleben gene bank collection comprises more than 150,000 plant accessions in total with more than 65,000 cereals that can be propagated and stored by seeds. However, beside mint and garlic accessions, almost 6,000 cultivated and wild species of potato needed to be maintained by alternative strategies.

The most widely practiced method for plant *ex situ* preservation of plant genetic resources is the storage of desiccated seeds at low temperature. Food plants mainly produce seeds that undergo maturation drying and are thus tolerant to desiccation. Seeds of this type are termed orthodox, and 90 % of the stored gene bank accessions are maintained as seeds. However, this traditional seed banking approach is not feasible for three different categories of crops: First group comprises crops like banana and plantain (*Musa spp*.), which do not produce seeds at all. Second group contains root and tuber crops like potato (*Solanum tuberosum*), yams (*Dioscorea spp*.), sweet potato (*Ipomoea batatas*) and sugarcane (*Saccharum spp.*).

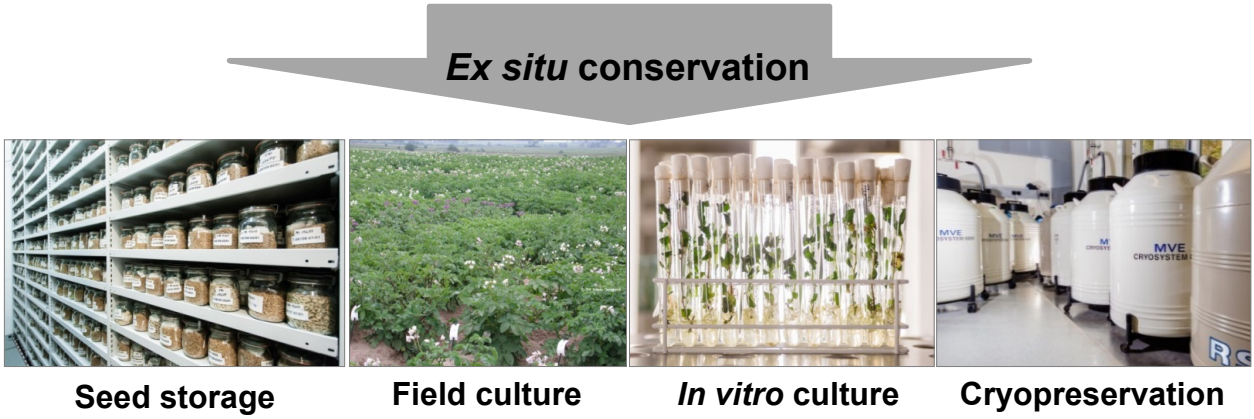



**Figure 1. Different strategies for *ex situ* germplasm maintenance for cereals (seed storage) and vegetatively propagated plant material like potato on field, *in vitro* or via cryopreservation.**

These crops also show sterile genotypes, whereas some produce small quantities of highly heterozygous orthodox seeds. The third group includes crops that produce seeds which do not undergo maturation drying and have a relatively high moisture content. Such seeds are unable to withstand desiccation and are often sensitive to chilling. Therefore, they cannot be stored under the conventional seed storage conditions described above, like low moisture content and low temperature. Seeds of this type are called recalcitrant and have to be stored in moist, relatively warm conditions to maintain viability. Species of predominantly tropical or subtropical origin, such as coconut, cacao and many forest and fruit tree species produce recalcitrant seeds (Engelmann and Takagi, 2000; Keller *et al.*, 2006; Panis *et al.*, 1996; Wu *et al.*, 2003). Crops belonging to these three groups, as they are listed above, are vegetatively propagated either on field, via *in vitro* culture or cryopreserved to maintain genetic resources for future generations.

Vegetative preservation via field bank collection is prone to extreme weather events, disease infestation, and climate change result in the loss of valuable germplasm. The maintenance is labor-intensive and costs for technical personnel are very high. Moreover, permanent field maintenance provokes the danger of mixing accessions by displacing of tillers (Engelmann, 1997; Lynch *et al.*, 2007).

Maintenance of *in vitro* propagated species is still labor-intensive and there is the risk of losing accessions due to bacterial and virus infection, somaclonal variation and epigenetic changes or human error (Kaeppler *et al.*, 2000; Keller *et al.*, 2006). Plants deals with different conditions in the microenvironment, like exogenous addition of plant growth regulators or humidity in the vessels (Gaspar *et al.*, 1996; Vanstraelen and Benková, 2012) and tissue and organ formation require coordination between genetics and epigenetics (Jaenisch and Bird, 2003; Smulders and de Klerk, 2011). In fact, the slow growth regimes enable subcultures to be extended up to four years for some plant species (Ashmore, 1997). However, some cultures of garlic clones for instance are not suitable for long-lasting *in vitro* propagation. The viability declines and bacterial infection decrease the quality of donor material (Keller, 2005). As a consequence the influence of *in vitro* storage on the germplasm has to be checked carefully and alternative approaches like cryopreservation needs to be introduced to long-term plant conservation strategies.

### 1.3. Plant cryopreservation

Cryopreservation at ultra-low temperature in liquid nitrogen (LN) at -196 °C or in the vapor phase at which temperature ranges between -136 and -180 °C (Day *et al.*, 2008) is one possible approach to assure the *ex situ* conservation of genetic resources. Especially germplasm which can only be reproduced vegetatively or selected clonal material/varieties of heterozygous plant species are frequently used (Keller, 2005; Panis *et al.*, 1996).

At extreme cool temperatures, metabolic, biochemical and most physical processes are arrested and thus the material can be stored for a theoretically unlimited period of time (Shibli *et al.*, 2004) with low cost and little space (Keller *et al.*, 2012). Cryopreservation is currently the only safe and cost-effective option for the long-term conservation of genetic resources of problem species. With plants, it is used for recalcitrant and intermediate seed species, vegetatively propagated plants, rare and endangered species, biotechnology products such as metabolite-producing cell lines and genetically engineered material. Nowadays, the largest collections of cryopreserved plant material in *ex situ* plant gene banks comprise dormant buds of apple at National Plant Germplasm System (NPGS) in USA, potato shoot tips at Leibniz-Institute of Plant Genetics and Crop Plant Research (IPK) Gatersleben in Germany and the Potato Cryobank at International Potato Center (CIP) in Peru, dormant buds of mulberry at National Institute of Agrobiological Science (NIAS) in Japan and NBPGR in India, as well as banana, cassava, elm, and garlic (**Table 1**).

Another aspect in the field of cryobiology is cryotherapy. Cryotherapy is based on the cell destruction by ice crystal formation during cryopreservation. Cryopreservation was applied to erase viruses from potato, banana or grape (Helliot *et al.*, 2002; Wang *et al.*, 2006b; Wang *et al.*, 2003).

**Table 1. Cryocollections of plant germplasm maintaining more than 300 accessions.**

| Species | Shoot tips | No. | Institute | Source |
|---|---|---|---|---|
| Apple | Dormant bud | 2155 | NPGS[1], USA | Jenderek and Reed (2015) |
| Potato | Shoot tips | 1560 | IPK[2], Germany | Nagel (2018) |
| Mulberry | Dorman bud | 1,236 | NIAS[3], Japan | Niino and Arizaga (2015) |
| Allium | Shoot tips | 1100 | NICS[4], Korea | Kim *et al.* (2012) |
| Potato | Shoot tips | 1028 | CIP[5], Peru | Vollmer *et al.* (2016) |
| Banana | Shoot tips | 900 | ITC[6], Belgium | www.fao.org |
| Cassava | Shoot tips | 480 | CIAT[7], Colombia | Niino and Arizaga (2015) |
| Elm | Dormant bud | 440 | AFOCEL[8], France | Niino and Arizaga (2015) |
| Mulberry | Dorman bud | 329 | NBPGR[9], India | Niino and Arizaga (2015) |
| Garlic | Shoot tips | 300 | NICS[4], Korea | Niino and Arizaga (2015) |

[1]NPGS: National Plant Germplasm System; [2]IPK: Leibniz-Institute of Plant Genetics and Crop Plant Research; [3]NIAS: National Institute of Agrobiological Sciences; [4]NICS: National Institute of Crop Science, [5]CIP: Cryobank at International Potato Center; [6]ITC: International Musa Transit Centre; [7]CIAT: International Center for Tropical Agriculture; [8]AFOCEL: Association Forêt Cellulose; [9]NBPGR: National Bureau of Plant Genetic Resources. http://www.fao.org/docrep/007/ae216e/ae216e03.htm

### 1.3.1. Theoretical basis of plant cryopreservation

Cryoinjury appears not during the long-term storage of germplasm in LN. Rather the progression to extreme cool temperature and back to room temperature (RT) results in ice crystal induced damage and affects post-cryogenic viability. Cryopreservation protocols, which have been optimized for a number of gene bank species, seek to avoid the formation of intra-cellular ice crystals in the tissue, as the ice expansion cause harmful, irreversible damage to cell membranes and destroy their semi-permeability (Engelmann, 2004).

In nature, plants evolved adaptation systems, where ice crystal formation at sub-zero temperature is reduced through the accumulation of cryoprotectants or anti-freeze proteins. The so-called hardening process induces the ability of plants to increase the osmotic value of the cell solutes. It is an adaptation in metabolism, which induces the accumulation of cryoprotectants like sugars or certain amino acids as proline and changes in lipid composition (Sieg *et al.*, 1996; Zuther *et al.*, 2004; Zuther *et al.*, 2012). Further, a number of cold-responsive genes encode hydrophilic LEA or LEA-like polypeptides are induced (Thomashow, 1999). An *Arabidopsis* cold-regulated gene, called *COR15a*, acts directly as a cryoprotective protein to prevent the formation of hexagonal II–phase lipids (Steponkus *et al.*, 1998).

Based on this principle, cryopreservation aims at concentrating the cell cytosol. Depending on germplasm and donor material, the cell cytosol is concentrated either through air drying, freeze dehydration or via the application of cryoprotectants like dimethyl sulfoxide (DMSO), sugars, sugar alcohols or additives like polyethylene glycol (PEG) (Bowler and Fuller, 1989). However, some cryoprotective agents like DMSO and glycerol were suspected to have toxic impact on cryopreserved tissue (Panis and Lambardi, 2005; Volk *et al.*, 2014).

In general, prepared plant tissue (phase I) is incubated in cryoprotectant solutions to reduce cell water content (phase II), prior to cooling in LN. After cryostorage, the germplasm is re-warmed, washed free of the cryoprotectants, and regenerated on recovery medium (phase III) (**Figure 2**).

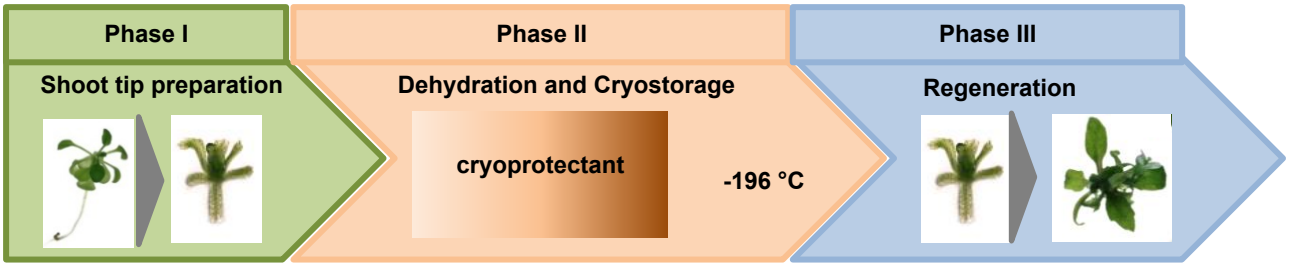



**Figure 2. Schematic overview of a long-term storage protocol.** Phase I: excision of shoot tips, phase II: gradual reduction in explant hydration by treatment with cryoprotectant, phase III: recovery from cryopreservation over 25 days.

### 1.3.2. Explants used for cryopreservation

Routine cryopreservation methods were developed for cell-suspension cultures (Withers and King, 1980). However, a range of plant tissues or organs are used as explants, including shoot tips, embryogenic cultures, buds, callus, pollen or cell suspension cultures (Benson *et al.*, 2007; Engelmann, 1991; Ogawa *et al.*, 2008; Panis and Lambardi, 2005; Reed, 2008).

Shoot tips bring several advantages and are the preferred explant in plant cryopreservation nowadays. Shoot tips are small in size (1-3 mm) and contain the shoot apical meristem (SAM) (**Figure 3**). Moreover, the meristematic tissue is composed of homogenous, actively dividing cells with small vacuoles. Plants generate new tissue through the activity of undifferentiated stem cells in the apical dome of the meristem. The use of meristematic tissues lowers the chance of somaclonal variation, resulting in a direct and organized recovery.

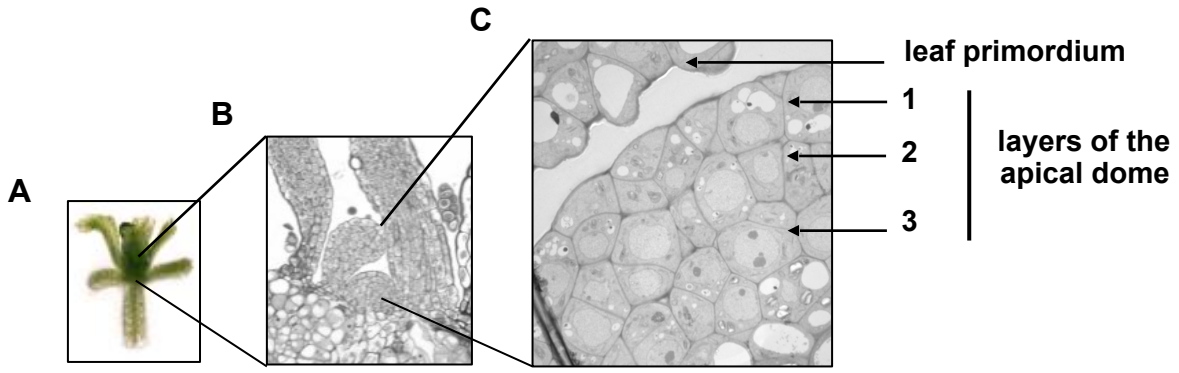



**Figure 3. A schematic view of an *Arabidopsis* shoot tip, showing the first three layers of the apical dome in the shoot apical meristem (SAM).** (**A**) Excised shoot tip; (**B**) Cross-section of SAM; (**C**) First three layers of SAM.

### 1.3.3. Plant cryopreservation techniques

During the last three decades, different cryopreservation methods have been established to increase the usage of cryogenic storage for plant genetic resources. Slow Cooling, Encapsulation/Dehydration, and Vitrification are predominantly used as cryopreservation methods. Each protocol was established for a distinct type of explant, with modified cooling speed, cryoprotectants and dehydration treatment (**Figure 4**).

Slow Cooling or Controlled Freezing was one of the first standard protocols for plant cryopreservation (Withers and King, 1980). Controlled Freezing induces dehydration of plant material before cryostorage in LN. This approach was mainly applied for non-organized tissues like callus or cell suspension. Culture is cooled to temperatures of

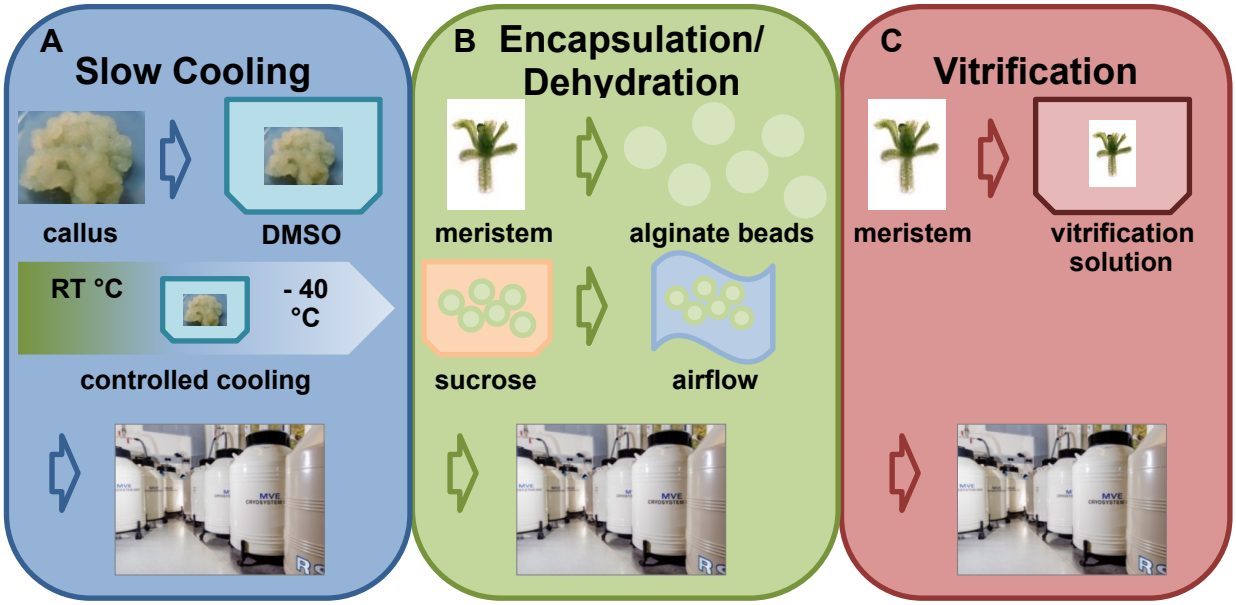



**Figure 4. Scheme of basic plant cryopreservation protocols. (A)** Slow Cooling. **(B)** Encapsulation/Dehydration. **(C)** Vitrification.

about -40 °C, at cooling rate of 0.5-2 °C/min, in the presence of cryoprotectant solutions like DMSO. Controlled slow cooling forces the formation of extracellular ice ahead of intracellular ice. Dehydration is caused by the outflow of water from the cells and after reaching this point tissues are transferred to LN (Kartha *et al.*, 1980; Reed, 1988) (**Figure 4A**).

Another strategy is the Encapsulation/Dehydration method, used for meristems or embryos (Fabre and Dereuddre, 1990). Plant material is encapsulated in alginate beads treated with high concentrated sucrose solutions and is finally dehydrated using airflow or silica gel prior LN storage (**Figure 4B**).

Vitrification combines the pre-treatment of germplasm with highly concentrated cryoprotectants and rapid cooling rates to obtain vitrification, a direct transition of water structure from liquid to the amorphous solid state (Mazur, 1984). For optimal vitrification process, a reduction in cell water content of at least 20-30 % is required. Rapid cooling rates (6 °C/sec) are obtained by plunging the explants enclosed in a cryovial into LN (**Figure 4C**). The most prominent cryoprotectant solutions are called Plant Vitrification Solutions (PVS). Nowadays PVS-based methods are most widely used because of their easiness in handling, high reproducibility and applicability to a wide range of plant species. PVS2 (Plant Vitrification Solution 2), the most common used solution, contains 30 % glycerol, 15 % ethylene glycol, 15 % DMSO and 0.4 M sucrose (Sakai *et al.*, 1990). Alternative vitrification solutions are PVS3 containing 50 % of glycerol and 50 % sucrose (Nishizawa *et al.*, 1993), PVS4 (Sakai *et al.*, 2000) or Steponkus` solution (Langis and Steponkus, 1990).

One adaptation to the Vitrification method is called Droplet Vitrification. It was developed by Kartha *et al.* (1982) and modified by Leunufna and Keller (2003). Shoot tips are treated with Plant Vitrification Solutions and then transferred in 5-10 µl droplets of PVS on aluminium foil, which are then put into cryovials prior LN treatment. The application of this method achieves higher cooling rates (130 °C/sec) because of the little volume of cryoprotectant solution in which the explants are placed (Panis and Lambardi, 2005; Towill and Bonnart, 2003). Droplet-Vitrification protocols are highly suitable for cryopreservation of different vegetatively propagated plant materials such as potato, mint, or garlic (Folgado *et al.*, 2014; Kim *et al.*, 2009; Senula *et al.*, 2007).

Further protocols were developed like Droplet Freezing (Schäfer-Menuhr *et al.*, 1997), or Encapsulation/Vitrification (Hirai and Sakai, 1999). However, these techniques have been applied only to a limited number of species.

Apart from attaining an optimal cryopreservation protocol that reduces ice damage, it is important to establish reproducible methods that can be applied across a broad range of genotypes. The wider application is restricted to obstacles like differential genotypic responses to cryopreservation, little knowledge with respect to causal factors in cryopreservation tolerance and bottlenecks in technology transfer.

## 1.4. Factors that impact cryopreservation

### 1.4.1. Quality of donor material

The high quality of the donor material is crucial for successful cryopreservation. Spores of microorganisms and hidden microbes like symbionts can contaminate the cultures. *In vitro* propagation and cryopreservation weaken the plants and might lead to an unexpected outbreak of endophytes (Kogel *et al.*, 2006). Even though the application of antibiotics eliminates and suppresses bacterial contamination of donor material, some endophytes are favorable or essential for plant growth and thus may be useful to preserve them together with the plant (Scherling *et al.*, 2009; Ulrich *et al.*, 2008).

### 1.4.2. Acclimation

Each plant species requires specific growth conditions, comprise precise temperature, light regime and storage period. Cryopreservation protocols mostly starts with a phase of preculture including cold treatment to improve recovery. This phase of cold acclimation or hardening increases the total concentration of total soluble sugars, but also phospholipids and antioxidants were promoted and influence the success of cryopreservation (Folgado *et al.*, 2015; Harding *et al.*, 2008). Cold acclimation of donor plants was especially beneficial for vitrification protocols. For potato shoot tips, it was shown that an acclimation phase increased the total concentration of soluble sugars, which was correlated with higher recovery (Kaczmarczyk *et al.*, 2008). Plants such as blackberry (Gupta and Reed, 2006), mint (Senula *et al.*, 2007), strawberry (Höfer and Reed, 2011) and *Arabidopsis* (Towill *et al.*, 2006) benefited from alternating temperatures in the growth phase before cryopreservation.

Recently, Edesi et al. (2017) showed that light spectral quality also affects regrowth of cryopreserved potato germplasm. Blue LED light enhanced recovery, whereas red LED light exposure resulted in lowest recovery.

### 1.4.3. Genotypic effects

Genotype dependence might affect the success of cryopreservation. Genotypes differ in morphological aspects like the size of the explant organs, but also in biochemical composition. Thus, it is hard to conclude from one species to another in a complex gene bank collection, where post-cryogenic recovery can vary from 10 % to 80 % like it was shown for potato accessions (Kaczmarczyk *et al.*, 2008).

### 1.4.4. Cryostressors

Cryopreserved plant tissues cope with a variety of different stress factors, so called cryostressors (**Figure. 5**). There are multiple changes in plant physiology, gene regulating and biochemical processes, which therefore potentially determines the success of post-cryogenic survival. Even though plants are not faced to cryostress in nature, the storage procedure imposes a spectrum of abiotic stresses. This includes wounding following the preparation of the explant from its mother plants, osmotic stress occurring as a result of the dehydration, and the chemical toxicity of the cryoprotectant itself. During cryostorage, the explants also suffer from a rapid and large variation in temperature, especially during the frequent transfer of cryovials and temperature fluctuations at the vapor phase.

These factors lead to the accumulation of reactive oxygen species (ROS) and make a significant impact on post-cryopreservation recovery. Membrane lipids are the primary targets for ROS (Benson *et al.*, 2004), and malondialdehyde (MDA) serves as

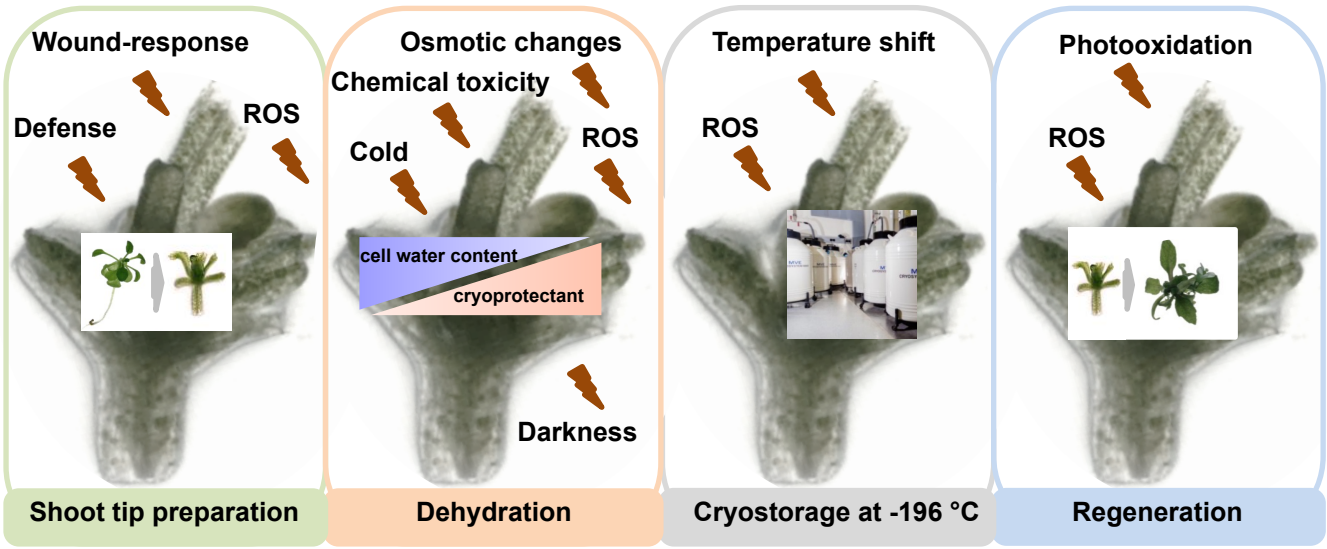



**Figure 5. Cryopreservation – a quantitative trait for different cryostressors.** Schematic overview of putative cryostressors (indicated with red flash) after shoot tip preparation, osmotic dehydration, cryostorage at -196 °C, and during the early phase of regeneration. ROS: reactive oxygen species.

a marker for high levels of lipid peroxidation in rice (Benson *et al.*, 1992), maize (Wen *et al.*, 2010) and *Arabidopsis* (Ren *et al.*, 2013). Consistently, elevated levels of antioxidants can improve the recovery of some plants (Lynch *et al.*, 2011; Volk, 2010).

However, ROS-induced lipid peroxidation seems to be the final effect of an imbalance in stress response towards cryostressors. Many stresses are accompanied by the excessive formation of ROS. Therefore, ROS are crucial to act as signaling molecules in plant stress response. ROS like superoxide, hydrogen peroxide and hydroxyl free radical are central components of the plant defense machinery. For instance, $H_2O_2$ the most long-lived and therefore major ROS of oxidative burst acts as a signaling molecule upstream of wounding response or pathogen attack (Nürnberger and Scheel, 2001). It remains unclear if increased ROS levels are cause or a result of cryostress, or both. Consequently, the molecular genetics of putative cryostressors need to be solved by using a model system of plant biology, to reveal putative targets of ROS signaling molecules.

## 1.5. *Arabidopsis* as model system in cryobiology

### 1.5.1. Advantages of a model system for cryopreservation

The knowledge of *ex situ* gene bank species, also in terms of cryopreservation associated factors, is much lower compared to well investigated model plants (Keller *et al.*, 2011). To get deeper insights in the molecular mechanisms underlying cryopreservation, *Arabidopsis* shoot tips can be used as an important tool to understand the basic reactions during the cooling and rewarming processes and support thereby the improvement of cryopreservation. The well-characterized genome, the existing diversity of wildtype accessions and availability of transgenic lines, facilitate the validation of stress-induced changes in relation to viability of cryopreserved *Arabidopsis*. Shoot tips can be successfully cryopreserved and regenerated independent from wildtype genotypes, which allows taking advantage of the extensive genomic and genetic resources developed for this species (Stock *et al.*, 2017; Towill *et al.*, 2006). *Arabidopsis* is in addition advantageous due to its high potential to obtain homogeneous seed-derived plant material where effects of pre-culture environment and endophytes can be mostly excluded.

Further, epigenetic effect based on changes in the DNA methylation pattern of *Arabidopsis* seedlings can be neglected. Li *et al.* (2013b) confirmed a genetic stability, whereas Wang and He (2009) determined changes by methylation-sensitive amplified

polymorphism technique. However, phenotypic traits, including stalk and flowering time, as well as yield-component traits of cryopreserved plants were normal, suggesting that variations were not inherited to the next generation.

### 1.5.2. Case studies in *Arabidopsis*

To date, only few studies dealt with the regulation of cryo-associated stress in *Arabidopsis*. Ogawa *et al.* (2008) started with the establishment of a routine protocol for suspension-culture cells. *Arabidopsis* cells were encapsulated, dehydrated and immersed in LN. High recovery and genetic stability confirm the suitability of this approach.

Almost one decade ago, differentially expressed genes during cryostress were identified in *Arabidopsis* shoot tips (Basu, 2008). From this study, drought induced genes showed evidence that cryogenic stored plant share common pathways with other abiotic stresses.

With the emergence of omics-approaches, microarray and RNA-*seq* datasets open the door to unravel regulatory mechanisms during cryopreservation (Chen *et al.*, 2015; Ren *et al.*, 2015; Ren *et al.*, 2013). Omics-based studies used *Arabidopsis* seedlings because they are easy to obtain, to handle and recover with high regeneration. Wang and He (2009) applied a vitrification based method for two days old *Arabidopsis* seedlings. After cryoprotection using PVS2, seedlings were immersed in LN, and finally recovered after cryostorage with regrowth up to 94 %. Ren et al. (2013) and Ren et al. (2015) found that regrowth can vary between 50 and 80 % and they stated that cryostorage imposes abiotic stress, particularly oxidative stress during cryoprotectant treatment. This finally affects post-cryogenic recovery, if the cryopreservation method is not optimized. As a consequence, induced lipid peroxidation needs to be reduced as a key for successful cryopreservation.

However, Ren *et al.* (2015) could also show that specific abiotic stress induced genes are involved in critical steps of cryopreservation. Especially, transcription factors of the AP2 (APETALA2)-ERF (ethylene responsive factor) family including *DREBs/ CBFs* were upregulated after cryoinjury, as well as transcription factors of the WRKY and MYB families are involved in the signaling cascade during cryo-induced stresses. In addition, *calcium-binding proteins*, and *OXI1* are key factors in ROS signal transduction by activating ROS producing and scavenging networks.

Recently, genes and processes relevant for the cryopreservation competence in *Arabidopsis* shoot tips were identified, including general abiotic stress marker, as well

as biotic stress genes. These include *SULFOQUINOVOSYLDIACYL GLYCEROL 1 (At4g33030; SQD*), *MULTIPROTEIN BRIDGING FACTOR 1 C (At3g24500; MBF1C*), a putative *ASPARTYL PROTEASE* (*At1g66180; ASP*), *PATHOGENESIS RELATED GENE 5* (*At1g75040; PR5*) and the gene encoding the *WRKY22* transcription factor (TF) (*At4g01250; WRKY22*) (Gross *et al.*, 2016).

### 1.6. Putative candidate genes involved in cryopreservation

Plants are sessile organisms and face changes in the environment, where many times abiotic stresses are accompanied by biotic stresses. Hence during evolution sophisticated signaling cascades and cellular response mechanisms have been developed to adequately cope with particular stresses or a combination of various environmental threats. Most stresses involve the formation of ROS, which need be detoxified by enzymatic and non-enzymatic compounds (Choudhury *et al.*, 2017; Suzuki *et al.*, 2012). Also the processes applied during cryopreservation are accompanied with a variety of abiotic stresses to the plant tissue (wounding, dehydration, chemical toxicity and large temperature fluctuations), and each step of the procedure could involve its own specific combination.

Plant adaptation to a particular stress includes a multitude of processes occurring in a time-dependent manner and affecting the expression of a large set of genes. Transcription is the first step and this process is regulated by transcription factors which result in either activation or repression of target genes.

More than 1500 TFs have been reported in *Arabidopsis* since 2000. They comprise a DNA binding domain which specifically recognizes the target DNA sequence and thus regulate gene expression (Mitsuda and Ohme-Takagi, 2009). In *Arabidopsis*, TFs are categorized into many groups according to the conserved binding domain (Rushton *et al.*, 2010). Transcription factor families such as WRKY play a unique role of plant-specific stress adaptation, regulating diverse responses through a sophisticated cross-communication (Bakshi and Oelmüller, 2014; Banerjee and Roychoudhury, 2015; Jiang *et al.*, 2017; Phukan *et al.*, 2016). Among others, target genes of WRKY TFs are *Pathogenesis Related Genes* (*PR*s). PRs comprise large and highly complex gene families involved in pathogen defense, as well as a wide range of developmental processes (Graham *et al.*, 2003; Hegde and Keshgond, 2013).

Therefore, *PR5* and *WRKY22* were promising candidates to reveal the processes induced by cryostress, which includes a wide range of different abiotic stressors.

### 1.6.1. PATHOGENESIS RELATED GENES

PR proteins are classified into 17 families based on their amino acid sequences, as well as their enzymatic or biological activity (Christensen *et al.*, 2002). For a number of PR proteins, activities are known or can be deduced and most of them possess antifungal activity. For instance PR1 is used as a marker for systematic acquired resistance response, PR2 showed β-1,3-glucanase activity, PR3, PR4, PR8 and PR11 showed chitinase function (Hegde and Keshgond, 2013; van Loon *et al.*, 1994). PR6, PR7, PR10, and PR12 are stable defensive proteins, whereas PR9 function as a peroxidase (Hegde and Keshgond, 2013; Liu and Ekramoddoullah, 2006; Vriens *et al.*, 2014). PR15 and PR16 catalyze oxidation of oxalates by molecular oxygen in plant defense (Caliskan, 2000).

Thaumatin-like proteins, like PR5, show antifungal activity, serve as glucanase, xylanase, α-amylase and trypsin inhibitors. They are especially induced during wounding and after the attack of phloem feeding insects (Dafoe *et al.*, 2010). *PR5* is involved in plant disease resistance linked to salicylic acid (SA), ethylene-mediated response, and oxidative burst (Chen *et al.*, 2010b; Kitajima and Sato, 1999; Li and Strid, 2005). Moreover, changes in *PR5* transcript expression after cutting *Arabidopsis* stems (Li and Strid, 2005), and drought stress response (Liu *et al.*, 2013) suggested an important role in shoot tip preparation and osmotic dehydration during cryopreservation.

Even though protein function of PR proteins started to be unveiled, gene expression of *PR* genes is not well investigated. Transcription regulation of *PR* genes involves interaction between transcription factors and cis-acting regulatory elements to enhance or repress spatiotemporal gene expression. These elements are short motifs situated in the promoter regions like TATA box, GC box, CAAT box (Kaur *et al.*, 2017; Kaur and Pati, 2016; Wittkopp and Kalay, 2011). Member of TF families, for instance the WRKY family, show binding preferences to a large variety of defense-related genes of the *PR* type (Eulgem *et al.*, 2000; Rushton and Somssich, 1998; Yang *et al.*, 1999).

### 1.6.2. WRKY transcription factor family

WRKY TFs belong to a very large gene family, represented in *Arabidopsis* by more than 70 members. The DNA binding domain of the WRKY transcription factors is termed as the WRKY domain because of the highly conserved WRKYGQK amino acid sequence. The WRKY domain constitutes a four-stranded β-sheet, is about 60 amino acid in length with the WRKY DNA binding domain sequence at the N-terminus and a

C-terminal $CX_{4-5}CX_{22-23}HXH$ zinc (zn) finger binding motif (Bakshi and Oelmüller, 2014; Eulgem *et al.*, 2000). Especially the Zn-finger motif is crucial for WRKY TFs to bind to W-box (C/T) TGAC (T/C) in the promotor of target genes. WRKY proteins are grouped based on the number of WRKY domains and the type of Zn-finger motif. Group I contains WRKY´s with two WRKY domains, whereas group II and III have only one WRKY domain but show differences in the amino acid composition of the Zn-finger like motif. WRKY transcription factors of the polyphyletic group II can be classified in IIa, IIb, IIc, IId, and IIe based on the primary amino acid sequence (Jiang *et al.*, 2017; Phukan *et al.*, 2016; Rushton *et al.*, 2010). They are involved in a wide array of functions, including defense, the abiotic stress response, senescence, development and hormone-mediated processes (Gao *et al.*, 2011; Liao *et al.*, 2016; Pecher *et al.*, 2014; Rushton *et al.*, 1996; Somssich and Hahlbrock, 1998; Weyhe *et al.*, 2014; Zhang *et al.*, 2015a).

*WRKY22*, belonging to the subgroup IIe, is known to regulate up-stream processes of low temperature acclimation (Chawade *et al.*, 2007; Park *et al.*, 2015), hypoxia-induced immunity (Hsu *et al.*, 2013), pathogen-triggered immunity (Dong *et al.*, 2003; Göhre *et al.*, 2012; Kloth *et al.*, 2016) and leaf senescence (Zhou *et al.*, 2011).

### 1.7. Objectives

The ultimate goal of this study was related to elucidate mechanisms relevant for cryostress tolerance. To prove whether *Arabidopsis* represents a suitable platform for identifying the basic responses to cryostressors, the main objectives were to:

- Establish a cryopreservation protocol for *Arabidopsis* shoot tips with high post-cryogenic recovery.
- Investigate thermal properties of cryopreserved tissue to exclude harmful ice crystal formation during the process of cooling and rewarming.
- Verify the approach with a large collection of *Arabidopsis* genotypes.

This study tackle fundamental research on plants stress defense system by analyzing the two candidate genes *PATHOGENESIS RELATED GENE 5* and the transcription factor *WRKY22*. By analyzing critical steps of cryopreservation (shoot tips preparation: phase I; PVS2 treatment: phase II; and early recovery: phase III):

- Basic molecular events occurring during the cryopreservation of WT *Arabidopsis* shoot tips were investigated.

## INTRODUCTION

- Putative cryostressors, induced during critical phases of cryopreservation were elucidated.
- Differences between the shoot tip transcriptomes of a *wrky22* KO mutant and WT were analyzed with the intention of detecting the downstream transcriptional effects associated with the presence of *WRKY22*.
- The putative role of *PR5* during the early phase (phase I) of cryopreservation dependent from the level of tissue rupture was analyzed.
- The interaction of *WRKY22* as a putative upstream-regulator of *PR5* during cryopreservation was verified.

# CHAPTER 2. MATERIAL AND METHODS

## 2.1. Plant material and growth conditions

For the establishment of the *Arabidopsis* shoot tips cryopreservation method, a set of well-characterized wildtype accessions Col-0, No-0, Sah-0, Edi-0, N-14, Bur-0, Ws-0, Hi-0, Ler-0, Ms-0, Ct-1, Rsch-4, Sf-2, Mt-0, Zu-0, Wil-0, Can-0, and Po-0, as well as Ms-0 and N-14 were used (Kover *et al.*, 2009; Zuther *et al.*, 2012). For all experiments, seeds of the *Arabidopsis* ecotype Columbia-0 (WT) were used as control. Relevant T-DNA insertion lines were obtained from the Arabidopsis Biological Resource Center (abrc.osu.edu), including SALK_098205 (*wrky22.1*), SALK_04720 (*wrky22.2*), SALK_058117 (*sqd*), SALK_079508 (*mbf1C*), SALK_025595 (*asp*), and SALK_055603 (*pr5*). Double knockout mutants (*wrky22*x*pr5*) were obtained by reciprocal crossing of single knockout mutant *wrky22.1* and *pr5*. Plants harboring T-DNA mutations in the F2 generation were validated by a genomic PCR with primer sequences shown in **Table 2**.

**Table 2. Gene specific primers used for tracking of T-DNA insertion lines.**

| Locus | Gene | Name | Sequence *5′ - 3′* | Tm [°C] |
|---|---|---|---|---|
| At4g01250 | *WRKY22* | SALK_098205_LP | *Fw* -CAC AGA ACC AGA AAC GTC CTC- | 59.8 |
| | | SALK_098205_RP | *Rv* -ATA TTC CTC CGG TGG TAG TGG- | 59.8 |
| | *WRKY22* | SALK_04720_LP | *Fw* -TAC TGC TGA CGG ATT CCG- | 57.9 |
| | | SALK_04720_RP | *Rv* -CCT TTA CCA AAA ATG TAA CGC AG- | 57.1 |
| At4g33030 | *SQD* | SALK_058117_LP | *Fw* -GAA TTG GCC AAT TGG GAT ATC- | 55.9 |
| | | SALK_058117_RP | *Rv* -CAC TTA ACC GGT TCT GTG TGC- | 59.8 |
| At3g24500 | *MBF1C* | SALK_079508_LP | *Fw* -TGT TTC TGG ACC AGG AAC ATC- | 57.9 |
| | | SALK_079508_RP | *Rv* -TTT CTC CAT TTT CGT CTC TGG- | 55.9 |
| At1g66180 | *ASP* | SALK_025595_LP | *Fw* -TCC ACA AGT CTT TCA ACA CGA G- | 58.4 |
| | | SALK_025595_RP | *Rv* -GAA CCT TCG CTG AGG GTA ATC- | 59.8 |
| At1g75040 | *PR5* | SALK_055603_LP | *Fw* -CAT TTC ATT AAT GGC TCG CTC- | 55.9 |
| | | SALK_055603_RP | *Rv* -ATT GCT TTA TGG CCA CAG AC- | 57.9 |
| | | LBb1.3_BP | ATT TTG CCG ATT TCG GAA C | 52.4 |

Full-length coding sequences of either *WRKY22* or *PR5* were amplified from cDNA with relevant primer sequences given in **Table 3.** cDNA was cloned into the binary vector pB2GW7.0 (Karimi *et al.*, 2002) under the control of the CaMV 35S promoter

(*35S:comp*) using the Gateway system (www.thermofisher.com), according to supplier's protocol. The vector was introduced into either the *wrky22.1* or *pr5* mutant using the *Agrobacterium*-mediated floral dip method (Bechtold *et al.*, 1993).

Successfully transformed plants were identified using BASTA selection, supported by a qRT-PCR assay directed at *WRKY22* or *PR5*. The relevant primer sequences are given in **Table 5** (section 2.5. qRT-PCR).

**Table 3. Primer sequences used for Gateway cloning.**

| Locus | Gene | Sequence *5′ - 3′* | Tm [°C] |
|---|---|---|---|
| At4g01250 | *WRKY22* | *Fw* -CAC CAT GGC CGA CGA TTG- | 58.2 |
| | | *Rv* -TCA TAT TCC TCC GGT GGT AGT- | 57.9 |
| At1g75040 | *PR5* | *Fw* -CAC CAT GGC AAA TAT CTC CAG- | 57.9 |
| | | *Rv* -TTA AGG GCA GAA AGT GAT TTC G- | 56.5 |

For *in vivo* $H_2O_2$ detection, the binary vector pH2GW7:C-roGFP2-Orp1 (provided by Prof. Dr. Markus Schwarzländer; Münster University) was introduced into WT (Col-roGFP2-Orp1) and *pr5* mutant (*pr5*-roGFP2-Orp1) background using the *Agrobacterium*-mediated floral dip method (Bechtold *et al.*, 1993). Successfully transformed plants were identified using hygromycin selection, supported by a gDNA PCR assay directed at roGFP2-Orp1 vector with the primer sequences (5' – 3'): *Fw*: GCT TCA CCA TGG TGA GCA AGG and *Rv*: AGC TGC ACG CTG CCG TCC T.

Surface-sterilized seeds were plated on solidified Murashige and Skoog (1962) medium (MS) containing 3 % (w/v) sucrose, were cold-treated for 3 days at 4 °C, and held for 21 days under an 8 h photoperiod (light intensity 150 µmol $m^{-2}$ $s^{-1}$) with a day/night temperature regime of 22/20 °C. After this, the seedlings were either kept at 22/20 °C (non acclimated) or shifted to a 22/8 °C (acclimated seedlings) regime for three weeks to provide the explants used for cryostorage.

## 2.2. Cryostorage and regeneration

For the establishment of the protocol, four different methods were compared. Shoot tips of 6-week old seedlings were prepared that leaves, as well as the major parts of the roots were excised. The remaining parts, containing the apical dome, underlying tissue and the youngest, unexpanded primordial leaf, were employed to establish a cryopreservation protocol for *Arabidopsis* shoot tips. The final protocol used for further

applications, namely PVS2 a Droplet Vitrification adapted from Towill et al. (2006) is described briefly:

After an overnight immersion of the excised shoot tips in liquid MS medium (pH 5.8) containing 0.1 M sucrose (preculture), the material was partially desiccated by its immersion in MS medium (pH 5.8) containing 2 M glycerol and 0.4 M sucrose for 20 min (dehydration). This solution was then replaced by PVS2 (30 % w/v glycerol, 15 % w/v ethylene glycol, 15 % w/v dimethyl sulfoxide, 0.4 M sucrose in MS, pH 5.8) for 1 h at 4°C in the dark, prior to placement in 2.5 µL droplets of PVS2 solution on small pieces of heat-sterilized aluminium foil (10 shoot tips/foil). The foils were transferred to 1.2 mL cryovials, snap frozen in LN and held there for at least 1 h. After treatment with liquid nitrogen, shoot tips were rewarmed in MS medium (pH 5.8) containing 1.2 M sucrose for 10 min and placed onto recovery medium.

The major differences between PVS2 a and the additional protocols are summarized in **Table 4** and briefly described in the following section. PVS2 b Droplet Vitrification protocol was established for *Mentha* germplasm cryopreservation (Senula *et al.*, 2007). Using this protocol, the dehydration phase was extended to 2 h, whereas the PVS2 treatment was shortened to 20 min. PVS3 Droplet Vitrification protocol was conducted with a PVS3 (50 % w/v glycerol and 50 % sucrose in MS liquid medium) incubated for 1 h at 4 °C (Towill *et al.*, 2006). DMSO Droplet Freezing protocol was adapted from Kaczmarczyk *et al.* (2008). Shoot tips were precultured with 0.1 M sucrose in MS liquid medium as described for the PVS-based methods. On the second day the shoot tips were placed in cryoprotectant solution (0.1 M sucrose and 10 % DMSO in MS liquid medium) for 2 h at RT. After liquid nitrogen treatment, the shoot tips were rewarmed in 0.1 M sucrose in liquid MS medium and for washed for 10 min.

**Table 4. Comparison of the key steps which vary among the different protocols.**

| Protocol | Dehydration | Cryoprotectant | Rewarming |
|---|---|---|---|
| **PVS2 a** [1] | 2 M glycerol and 0.4 M sucrose **for 20 min** | PVS2 for **1h** | 1.2 M sucrose for 10 min |
| **PVS2 b** [2] | 2 M glycerol and 0.4 M sucrose **for 2 h** | PVS2 for **20 min** | 1.2 M sucrose for 10 min |
| **PVS3** [1] | 2 M glycerol and 0.4 M sucrose **for 20 min** | PVS3 for 1h | 1.2 M sucrose for 10 min |
| **DMSO Droplet** [3] | 0.1 M sucrose and 10 % dimethyl sulfoxide for 2h | | **0.1 M sucrose** for 10 min |

Adapted from [1]: Towill *et al.,* 2006; [2]: Senula *et al.,* 2007; [3]: Kaczmarczyk *et al.,* 2008.

For regeneration, the explants were placed on recovery medium (0.1 M sucrose, 0.5 mg $L^{-1}$ zeatin riboside, 0.2 mg $L^{-1}$ gibberellic acid ($GA_3$), 0.5 mg $L^{-1}$ indole-3-acetic acid, 1 % agar in MS medium, pH 5.8) and maintained in the dark for three days at 22 °C, then for four days under low light, long-day conditions (16 h photoperiod, irradiance 20-30 µmol $m^{-2}$ $s^{-1}$, 22/20 °C), and finally under a normal light regime (16 h photoperiod, irradiance 150 µmol $m^{-2}$ $s^{-1}$, 22/20 °C) for additional 18 days. Visual assessments were made after a recovery period of 25 days: explants showing no sign of any development were considered as “dead”, while those which regenerated not complete developed shoot/root/leaf structures or callus were classed as “surviving”; the third category represented those which developed into normal plants (“recovered”). Only recovered plantlets were included in the statistical analyses, which were based on the Win Fisher test. The values reported here represent the mean of three replicates, each of which comprised a group of 30 shoot tips. Recovery represents mean percentage value of portion with absolute standard deviation (Fagan, 1996). In parallel to each cryopreservation experiment, shoot tips went through the protocol without LN treatment (LN-) as a control. Explants always recovered to almost 100 %.

To verify that recovered plantlets were still able to produce seeds, regenerating plantlets were planted in the greenhouse after PVS2 a treatment. Two weeks after cryopreservation, about 10 % of recovered plantlets were transferred to soil in a growth cabinet. After 3 weeks under short day conditions (8 h photoperiod, irradiance 150 µmol $m^{-2}$ $s^{-1}$, 22/20 °C), day length was increased to 14 h photoperiod for at least 8 weeks. Dried siliques of mature plants were harvested and the produced seed material was stored at 4 °C for subsequent experiments.

## 2.3. Differential scanning calorimetry

Differential scanning calorimetry belongs to thermal analysis methods that can be used for measurements and determination of glass transitions and crystallization in cryopreservation. A Differential Scanning Calorimeter (DSC) Q2000 including a LN cooling system (TA Instruments - Waters, New Castle, Delaware, USA) was used to perform thermal analyses of *Arabidopsis* Col-0 shoot tip cell water content for the individual protocols. Three to five acclimated shoot tips were pooled per DSC run. Measurements were performed after cryoprotectant treatment with PVS2 a and b, PVS3, or DMSO (**Table 4**). Samples were weighted on a Sartorius microbalance ME235S (Sartorius, Göttingen, Germany) and placed in hermetically sealed 40 µl

aluminium pans. Measurements were conducted using the following parameters. The DSC was set to a cooling rate of 10 °C/min to -140 °C, isothermal annealing at -140 °C for 5 min, and rewarming rate of 10 °C/min to 20 °C. Each experiment was repeated three times. Thermograms were analyzed by Universal Analysis 2000 Version 4.3A (TA Instruments - Waters, New Castle, Delaware, USA).

### 2.4. Standard molecular techniques

The standard molecular techniques such as polymerase chain reaction (PCR), agarose gel electrophoresis and transformation of *Escherichia coli* and *Agrobacterium tumefaciens* strains, were carried out according to Sambrook *et al.* (1989). Elution and purification of DNA fragments from the gel was performed using the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's protocol. Plasmid DNA purification from *E. coli* was carried out using the QIAprep Miniprep Kit (Qiagen) according to the producer's protocol. Oligonucleotides were synthesized and purified by Eurofins Genomics GmbH (Ebersberg, Germany). All cloning steps were performed using *Escherichia coli* XL1 Blue strain, and transformation of *Arabidopsis* plants or protoplasts of *Nicotiana benthamiana* was done using *Agrobacterium tumefaciens* GV2260 (Deblaere *et al.*, 1985).

### 2.5. RNA extraction and qRT-PCR

RNA extraction from three to five shoot tips after each step of cryopreservation was performed using an RNeasy Plus Micro Kit (Qiagen GmbH, Hilden, Germany). A 0.5 µg aliquot of DNase I-treated RNA was used as the template for synthesis of the first cDNA strand, using Maxima Reverse Transcriptase, primed by oligo $(dT)_{18}$ (Thermo Fisher Scientific, Waltham, MA, USA). The resulting cDNAs were subjected to qRT-PCRs driven by a variety of gene-specific primers (**Table 5**) in reactions based on SsoAdvanced™ Universal SYBR® Green Supermix (BioRad Laboratories, Hercules, CA, USA). The amplifications were run on a LightCycler® 480 Real Time PCR System (Roche Diagnostics GmbH, Mannheim, Germany), with three technical replicates. The resulting data were analyzed using QBASEPLUS v2.3 software (Biogazelle, Ghent, Belgium), employing the genes *CLATH* (*At5g46630*) and *TIP41* (*At4g34270*) (Czechowski *et al.*, 2005) as housekeeping sequences (primer sequences given in **Table 5**). Primer specificity was assessed by inspection of a melting curve derived after 40 amplification cycles.

**Table 5. List of quantitative RT-PCR primer sequences.**

| Locus | Gene | Sequence *5′ - 3′* | Tm [°C] |
|---|---|---|---|
| AT5G46630 | *CLATH* [1] | *Fw* -GCC AAT GTT CAC AGC ATC TGG TC- | 62,4 |
| | | *Rv* -ACC GCT CTT CTC CCA AAC CTT G- | 62.1 |
| AT4G34270 | *TIP41-like* [1] | *Fw* -ATG AAC TGG CTG ACA ATG GAG TG- | 60.6 |
| | | *Rv* -GAG CTT GGC ATG ACT CTC AC- | 59.4 |
| At1g75040 | *PR5* | *Fw* -AAA TAT CTC CAG TAT TCA CAT TC- | 53.5 |
| | | *Rv* -AAG TCT GTG GCC ATA ACA GCA A- | 58.4 |
| AT4G01250 | *WRKY22* [2] | *Fw* -CGT CCT CTT TCT CTC TCT GCT TCT T- | 63.0 |
| | | *Rv* -CCA TGC CCA GAC ATC GGA GTT TA | 62.4 |
| AT3G12860 | *NOP-56 like* | *Fw* -AAT CAC CCT CCG GCT ACG G- | 61.0 |
| | | *Rv* -CTC AGC TCA TCG CTC ATG TAT C- | 60.3 |
| AT3G22660 | *EBP2* | *Fw* -ATG TCA TTG GAA GAG GAT ATA GTA TCA- | 60.7 |
| | | *Rv* -AGT CCA ATC AAC ATC TTC AGG CCA- | 61.0 |
| AT3G05060 | *NOP-58 like* | *Fw* -CCT ATG AGC TTG GGT CTG TCT- | 59.8 |
| | | *Rv* -TGA TAT GAT CTT AGC AAG CTC GG- | 58.9 |
| AT5G38410 | *RBCS3B* | *Fw* -CGC AAC AAG TGG ATT CCT TGT- | 57.9 |
| | | *Rv* -AAT GAG CAG AGA TAA TTC ATA AGA ATG- | 57.4 |
| AT2G39730 | *RCA* [3] | *Fw* -TCG TTG AGA GCC TTG GAG TT- | 57.3 |
| | | *Rv* -CTG AGG TAG GTC TCG GCA A- | 58.8 |
| AT5G64040 | *PSAN* | *Fw* -AAT ACC TCG AGA GGA GCA AAA C- | 58.4 |
| | | *Rv* -AAA GCA ATA TCT TCT GAG ATA AAT GGA A- | 57.8 |

[1] Czechowski et al., 2005; [2] Hsu et al., 2013; [3] Zanten et al., 2014.

### 2.6. RNA-*seq* analysis and accession number

For the purpose of the RNA-*seq* analysis, RNA extraction from shoot tips at each of the three cryostress phases: at the end of phases I, II and the first day of regeneration was performed. RNeasy Plant Mini Kit (Qiagen) was used from a bulk of 100 shoot tips per replicate (three) per sampling point from both WT and *wrky22.1* mutant. mRNA purification and polyA selection was performed with DNase treated RNA using the Illumina TruSeq RNA Sample Preparation v2 Kit (Illumina, San Diego, California, USA). Library preparation was performed using ScriptSeq™ v2 RNA-*seq* Library Preparation Kit (Epicentre, Madison, Wisconsin, USA) following manufacturer's protocol. Quality assessment of the libraries was done using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, California, USA). Cluster generation of the prepared

libraries was performed using the cBot (Illumina) and TruSeq SR Cluster Kit v3–cBot-HS (Illumina) following manufacturer's instructions. The concentration of libraries loaded in the flowcells was 12 pM, followed by sequencing on a HiSeq 2500 instrument with the TruSeq SBS Kit v3 - HS (Illumina) for 50 cycles. Image analysis and base calling were performed using the Illumina pipeline v 1.8.

RNA sample preparation, library preparation, and sequencing was conducted by Dr. Boyke Bunk (Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures, Braunschweig)

The RNA-*seq* data from this work have been deposited to the European Nucleotide Archive (http://www.ebi.ac.uk/ena/submit) with the accession number PRJEB22967.

### 2.7. Read mapping and gene expression profiling, GO term enrichment analysis and MapMan functional annotation

Single end reads of triplicated WT and KO samples were mapped onto representative *A. thaliana* transcripts (TAIR v10; https://www.arabidopsis.org/) with kallisto (-l 190, -s 20) (Bray *et al.*, 2015). The estimated counts and calculated transcripts per million reads (TPM) were combined and analyzed using R software (www.r-project.org/). DEGs were determined using edgeR (www.bioconductor.org/packages/release/bioc/html/edgeR.html) (Robinson *et al.*, 2010), followed by multiple hypothesis testing correction (Bonferroni, 1936) to strictly avoid false positives at the possible expense of power. Gene annotations and ontology were retrieved from TAIR v10 and functional annotations from the MapMan repository (http://mapman.gabipd.org/) (**Appendix Data Set 3**). GO term enrichment analyses, were conducted with topGO (www.bioconductor.org/packages/ devel/bioc/html/topGO.html) (Alexa and Rahnenfuhrer, 2010) based on Fisher's Exact Test (Fisher, 1922).

Read mapping and gene expression profiling, and GO term enrichment was done by Prof. Andrea Bräutigam (Bielefeld University).

### 2.8. Phenotypic analysis

#### 2.8.1. Changes in stomatal aperture

A method adapted from Li *et al.* (2013) was used to estimate changes in stomatal aperture from leaves detached from four week old WT, *wrky22.1* and *wrky22.2* mutant plants. Estimates of stomatal closure were based on observations taken from three biological replicates. The leaves were floated in 30 mM KCl, 10 mM MES-KOH (pH 6.1)

under 150 µmol $m^{-2}$ $s^{-1}$ light for 2 h at RT, then in the same buffer containing either 10 µM ABA for 2 h at RT, or PVS2 at 4°C for 1 h in the dark. Stomatal aperture was represented by the ratio between the stomatal width and length, obtained from a sample of 80 stomata. Stomatal aperture was recorded with a Keyence Digital Microscope VHX-5000 (KEYENCE GmbH, Neu-Isenburg, Germany).

### 2.8.2. Drought stress experiment

A set of 40 plants of each of WT and the *wrky22.1* and *wrky22.2* mutants was soil-grown under an 8 h photoperiod (light intensity 150 µmol $m^{-2}$ $s^{-1}$) with a day/night temperature regime of 22/20 °C for four weeks, then transferred into a cabinet delivering an 8 h photoperiod (light intensity 120 µmol $m^{-2}$ $s^{-1}$), a temperature regime of 22/20 °C and a relative humidity of 40 %. Each pot was initially well-watered, after which water was withheld up to three weeks. At each sampling point, the fresh weight (FW) of three plants per genotype was obtained and their rosette diameter measured. Soil moisture was determined using a HH2 Moisture Meter (DELTA-T DEVICES, Cambridge, England) and the measurement of fresh weight and the rosette diameter.

## 2.9. Transient transformation of the 35S:GFP-PR5 construct and protoplast extraction

Full-length coding sequence of *PR5* without start codon was amplified from cDNA and cloned into to the binary vector pB7WGF2.0 (Karimi et al., 2002). As described in Mustroph *et al.* (2007), *A. tumefaciens* culture harboring 35S:GFP-PR5 construct was grown overnight in YEB medium with spectinomycin, rifampicin, 1 mM MES (pH 5.6) and 20 µM acetosyringone. Harvested pellet was washed twice and re-suspended in infiltration buffer (10 mM $MgCl_2$, 10 mM MES and 0.1 mM acetosyringone) to a final concentration of $OD_{600}$ of 1.0 and incubated at RT for 4 h. The suspension was infiltrated at the lower side of *Nicotiana benthamiana* leaves and after 2 d of incubation, leaf discs (7 mm in diameter) were harvested using a cork borer.

Protoplasts were isolated via incubation in K3AS medium (1xMS, 3 mM $CaCl_2$, and 0.4 M sucrose pH 5.8) dosed with 1 % cellulose and 0.2 % maceroenzyme for 4 h in the dark. Released protoplasts were centrifuged at 200 x g for 20 min at 4 °C and intact protoplasts were dilutes in 4 x volume of W5 medium (0.15 M NaCl. 0.13 M $CaCl_2$, 5 mM KCL, and 5 mM glucose; pH 5.8). Protoplasts were centrifuged for 5 min at 200 x g and the pellet was used for GFP analysis.

### 2.10. Confocal Laser Scanning Microscopy (CLSM)

GFP signals were detected using a confocal laser scanning microscope (LSM780, Carl Zeiss).

#### 2.10.1. Subcellular PR5 localization

For subcellular localization of PR5 fused to GFP, leaf protoplasts from *Nicotiana benthamiana,* harboring 35S:GFP-PR5, were isolated. Fluorescence of GFP was probed with a 488 nm laser line and fluorescence was recorded between 491 – 535 nm.

#### 2.10.2. Hydrogen peroxide detection via roGFP2 *in vivo* sensor

For *in vivo* $H_2O_2$ detection the protocol established by Schwarzländer et al. (2008) was adapted and described in the following section.

Six weeks old seedlings expressing pH2GW7:C-roGFP2-Orp1 were placed on a microscope slide. The slide was prepared with tape that the cover slide could not touch the seedling during measurements. The sample was covered with ½-strength MS medium (pH 5.8). The slide was mounted on the CLSM equipped with lasers for 405 and 488 nm excitation. Images were collected with a 25X lens in multi-track mode with line switching between 488 nm illumination and 405 nm illumination in ten replicates. The roGFP2 fluorescence was collected with a 505–530 nm emission band-pass filter. For the standard *in situ* calibration, the sample was covered with 100 mM $H_2O_2$ in ½-strength MS-medium for approximately 10 min, washed with ½-strength MS-medium (pH 5.8) for 1 min and then covered with freshly prepared 100 mM DTT in ½-strength MS-medium for 10 min. Each experiment included an internal calibration at the end of the experiment by perfusion with 100 mM $H_2O_2$ and 100 mM DTT to drive the roGFP2-Orp1 to the oxidized or the reduced form, respectively.

The ratio of excitation at 405/488 nm was calculated to monitor the accumulation of $H_2O_2$ in respond to shoot tip preparation (phase I), as well as preculture, dehydration and PVS2 treatment (phase II).

### 2.11. Transmission electron microscopy (TEM)

*A. thaliana* apical shoot tips (2-3 mm in length) were fixed in glutaraldehyde and osmium tetroxide, dehydrated by passing through an acetone series, embedded in polymerized Spurr resin, and polymerized in a heating cabinet. A detailed protocol for the fixation, substitution and resin embedding steps is given in the appendix section

(**Appendix: Table 1**). Ultra-thin sections (~70 nm) were cut using an Ultracut UCT microtome (Leica Microsystems, Vienna, Austria) and an ultra-diamond knife, transferred onto a transmission electron microscopy grid and contrasted using Leica EM stain containing uranyl acetate and Reynolds' lead citrate, before being subjected to transmission electron microscopy, using a Tecnai Sphera $G^2$ device (FEI, Eindhoven, The Netherlands), running at 120 kV.

The preparation of the shoot tip sections and analysis using transmission electron microscope were done by Dr. Michael Melzer (IPK, Gatersleben).

### 2.12. Statistical analyses

Analysis of variance (ANOVA) provided by the SigmaStat software was used for statistical analysis of data. In case of significant impact of the factor, Holm-Sidak or Bonferroni were conducted at $P \leq 0.05$. Comparison between the treatments and controls was carried out by use of the Student's t-test at $P \leq 0.05$. Used test is indicated in the figure legends, respectively.

### 2.13. Promotor *in silico* analysis

Prediction of putative promotor region and cis binding elements for *PR5* was conducted using PlantPan software (http://PlantPAN2.itps.ncku.edu.tw).

# CHAPTER 3. RESULTS

## 3.1. A simple method for cryopreservation of shoot tips of *Arabidopsis* genotypes

The aim of the presented study in this section (3.1) was the establishment of a cryopreservation protocol for *Arabidopsis* shoot tips, independent of genotypes and to use this as a platform for further molecular analysis in *Arabidopsis* shoot tip cryopreservation.

### 3.1.1. Acclimated shoot tips treated with Plant Vitrification Solution 2 showed highest recovery

*Arabidopsis* WT (Col-0) shoot tips, including either an acclimation phase (acc.) or without acclimation (n. acc.), were treated with four different cryopreservation protocols. The viability of shoot tips (acc. and n. acc.) treated with either PVS2 a, PVS2 b or PVS3 was significantly higher compared to shoot tips treated with DMSO Droplet Freezing (**Figure 6**). 93 % of the explants (n.acc.) exposed to PVS2 a and 66 % of the

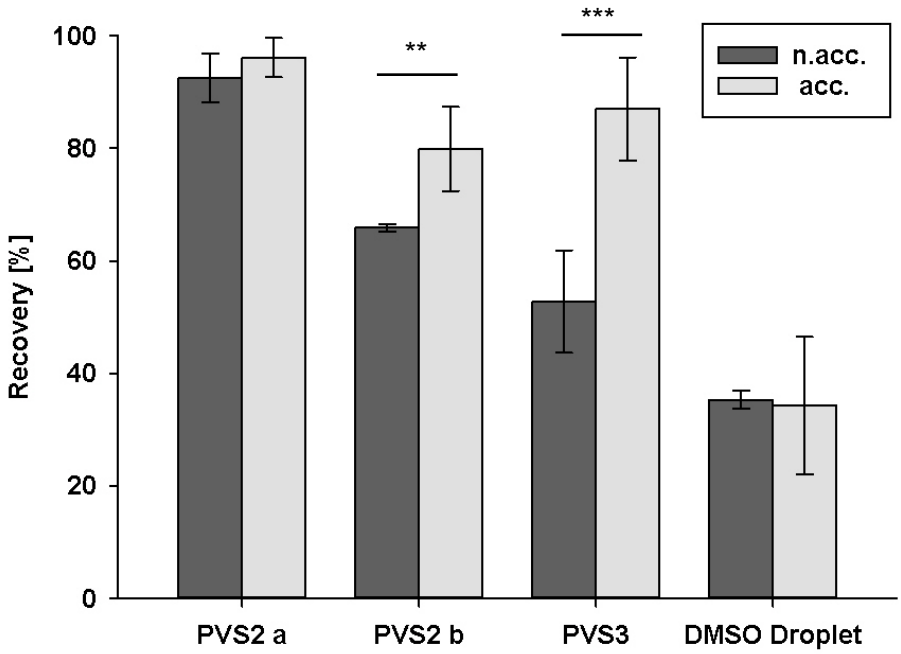



**Figure 6. Recovery of Col-0 (WT) plantlets depending on the used cryopreservation methods.** PVS2 a: PVS2 vitrification method; PVS2 b: PVS2 vitrification method for *Mentha* germplasm; PVS3 vitrification method; DMSO Droplet: DMSO Droplet Freezing method. n. acc.: non acclimated seedlings (22/20 °C); acc.: acclimated seedlings (22 °C/8 °C). Statistical significance was calculated using Win Fisher test comparing acclimated with non acclimated shoot tips (*** $P \leq 0.001$; ** $P \leq 0.01$; n≥60).

explants (n.acc.) exposed to PVS2 b recovered, whereas shoot tips (n.acc.) treated with PVS3 showed reduced viability of 53 %.

Recovery for shoot tips treated with the DMSO Droplet Freezing protocol dropped below 40 %, with and without acclimation, respectively. For two vitrification-based protocols, namely PVS2 b and PVS3 Droplet Vitrification, recovery could be significantly increased by including a hardening phase during the growing period of donor plants (acc.). Acclimation of shoot tips resulted in a recovery of 96 % for PVS2 a, 80 % for PVS2 b and 87 % for PVS3.

### 3.1.2. Plant vitrification solution protocols revealed glass transition during the cooling process

DSC measurements were conducted to investigate the thermal phase transition of water, and revealed a glass transitions during the cooling and rewarming process for the PVS-based protocols. Typical DSC thermograms were depicted in **Figure 7**, showing either a glass transition event (**Figure 7A**) or clear crystallization and melting (**Figure 7B**) during cooling and rewarming. Exothermic reactions are shown during cooling as positive heat flow (upper part of curve). Endothermic reactions are shown during rewarming as negative heat flow (lower part of the curve).

Glass transition onsets and specific heat capacities were similar for both PVS2 protocols (**Table 6**). Glass transition onset started at -116.50 ± 0.98 °C with a specific heat capacity of 1.09 ± 0.02 J/g* °C for shoot tips exposed to the PVS2 a protocol. For PVS2 b treated shoot tips glass transition started at -117.14 ± 0.49 °C with a specific heat capacity of 1.15 ± 0.05 J/g* °C. The PVS3 protocol exhibited a glass transition starting at -92.86 ± 0.29 °C with a specific heat capacity of 0.92 ± 0.01 J/g* °C. No glass transition event was detectable by using the DMSO Droplet-Freezing protocol. However, a melting onset was measured at -11.1 ± 1.2 °C with a melting enthalpy of 200.6 ± 0.8 J/g.

In conclusion, the appearance of a glass transitions during the cooling and rewarming process could be correlated with high recovery in WT shoot tips. To confirm that high recovery of *Arabidopsis* shoot tip is independent from genotype, a range of different wildtype accessions was screened towards their post-cryogenic capacity. Therefore, the most promising cryopreservation method, the PVS2 a Droplet Vitrification protocol including acclimation phase, was used for further experiments.

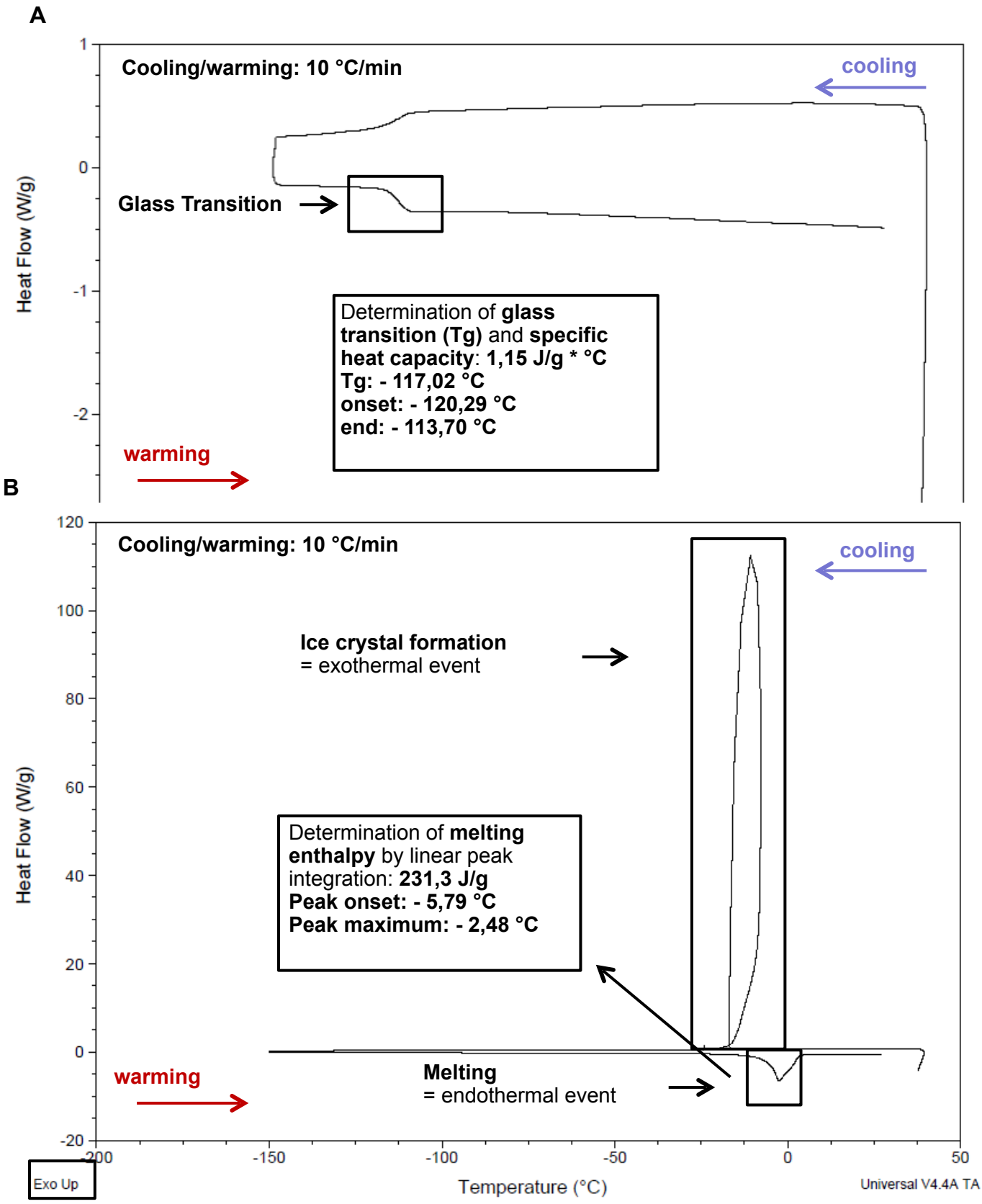



**Figure 7**. **Example of DSC thermograms** of (**A**) glass transition events or (**B**) crystallization and melting event. Calculated specific heat capacity and melting enthalpy is indicated in black rimmed boxes. Speed of cooling and warming: 10 °C/min.

**Table 6. Crystallization and glass transition parameters (mean ± SD) of *Arabidopsis* shoot tips (Col-0) after cryoprotectant treatment (PVS2/PVS3/DMSO).** Transitions were measured in the warming scan. Cooling and warming scans were performed at 10 °C/min.

| Glass Transition | | |
|---|---|---|
| Treatment | Onset [°C] | Specific Heat Capacity [J/g* °C] |
| PVS2 a | -116.50 ± 0.98 | 1.09 ± 0.02 |
| PVS2 b | -117.14 ± 0.49 | 1.15 ± 0.05 |
| PVS3 | -92.86 ± 0.29 | 0.92 ± 0.01 |
| **Crystallization** | | |
| | Melting Onset [°C] | Melting Enthalpy [J/g] |
| DMSO Freezing | -11.1 ± 1.2 | 200.6 ± 0.8 |

### 3.1.3. PVS2 Droplet Vitrification revealed high recovery across the range of *Arabidopsis* accessions

Among all tested *Arabidopsis* accessions, extremely high recovery could be obtained, by using the PVS2 a protocol including an acclimation period, as depicted in **Figure 8.** The highest recovery was reached for the No-0 wildtype with 99 % of recovered plantlets. The lowest was detected for the Po-0 accession, for which 89 % of the shoot tips recovered. Based on recovery %, three significantly different types of cryopreservation responses could be observed. Group I contained wildtype accessions, which achieved similar recovery compared to Col-0, namely No-0, Sah-0, and Edi-0 with an average recovery of 99 %. Accessions of Group II showed a moderately decreased recovery compared to Col-0 with an average of 95 % and included the wildtype accessions N-14, Bur-0, Ws-0, Hi-0, Ler-0, Ms-0, Ct-1, and Rsch-4. Group III included the accessions with significantly decreased recovery compared to Col-0 with an average of 91 % recovery. Group III contained Sf-2, Mt-0, Zu-0, Wil-0, Can-0, and Po-0 (**Figure 8A**). However, to evaluate whether recovering plantlets were still fertile and able to produce seed material, about 10 % of the viable plantlets of each genotype were transferred to soil. Almost 100 % plantlets of all groups regenerated and formed shoots and flowers for later seed development (**Figure 8B**).

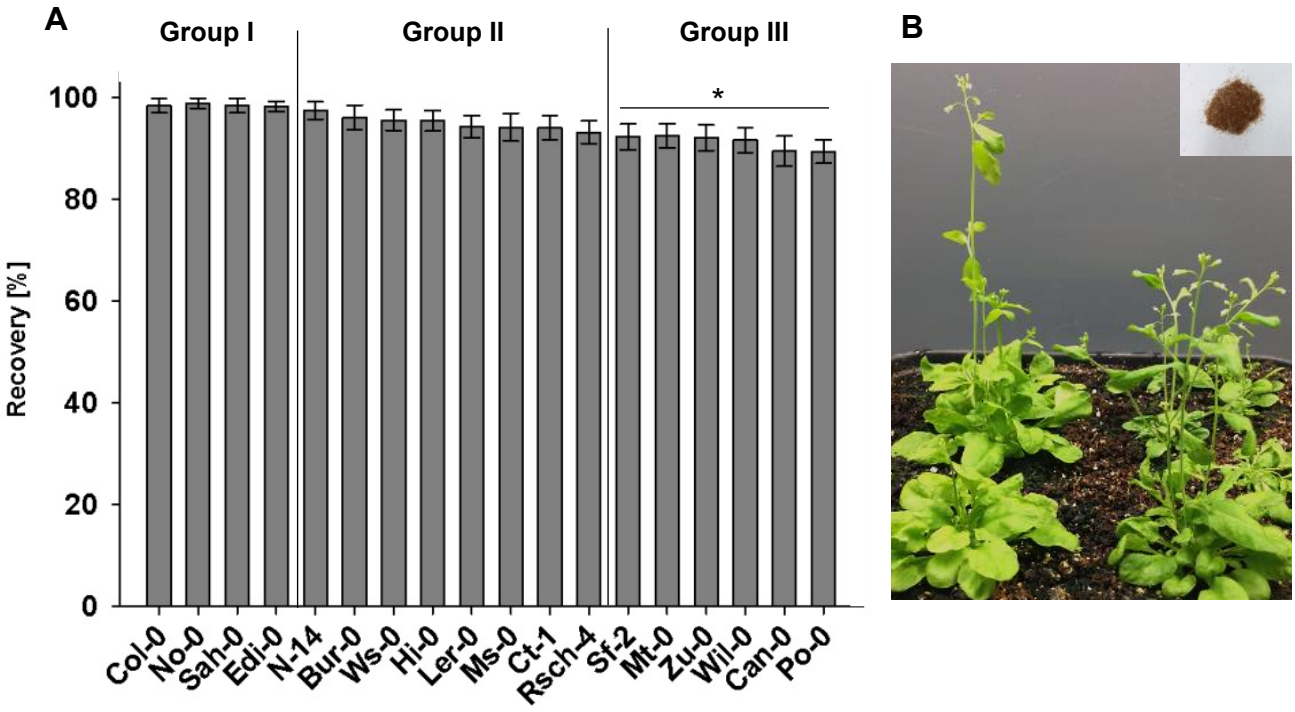



**Figure 8. Recovered plantlets after PVS2 Droplet Vitrification (PVS2 a). (A)** Percentage of recovered plantlets 25 d after cryopreservation among different wildtype accessions. Bar represents mean percentage value of portion with standard deviation. Statistical significance was calculated using Win Fisher test comparing wildtype accessions to Col-0 (* $P \leq 0.05$; n≥80). **(B)** *Arabidopsis* Col-0 plants after additional 10 weeks in pots and harvested seed material.

### 3.2. T-DNA mutant studies showed reduced regrowth of cryopreserved explants

Section 3.1. showed that the PVS2 Droplet Vitrification protocol presents a stable and genotype-independent *Arabidopsis* cryopreservation protocol. To get deeper insights into the molecular background of cryostress putative candidate genes (Gross *et al.*, 2016) were analyzed. The effect of knocking out genes on the performance of cryopreserved explants was explored by exposing T-DNA knockout lines for each gene to the PVS2 a cryostorage protocol (**Figure 9**). Candidate genes were the biotic stress genes: *PATHOGENESIS RELATED GENE 5* (*pr5*) and the *WRKY22* transcription factor *(wrky22*); a gene involved in the sulfolipid biosynthesis: *SULFOQUINOVOSYLDIACYL GLYCEROL 1* (*sqd*); and abiotic stress genes: *MULTIPROTEIN BRIDGING FACTOR 1 C* (*mbf1C*), and a *putative ASPARTYL PROTEASE* (*asp*). The vast majority (98 %) of WT shoot tips regenerated into viable plantlets after cryogenic treatment, as did explants from the mutant lines involving *sqd*, *mbf1C* and *asp*. In contrast, the lack of a

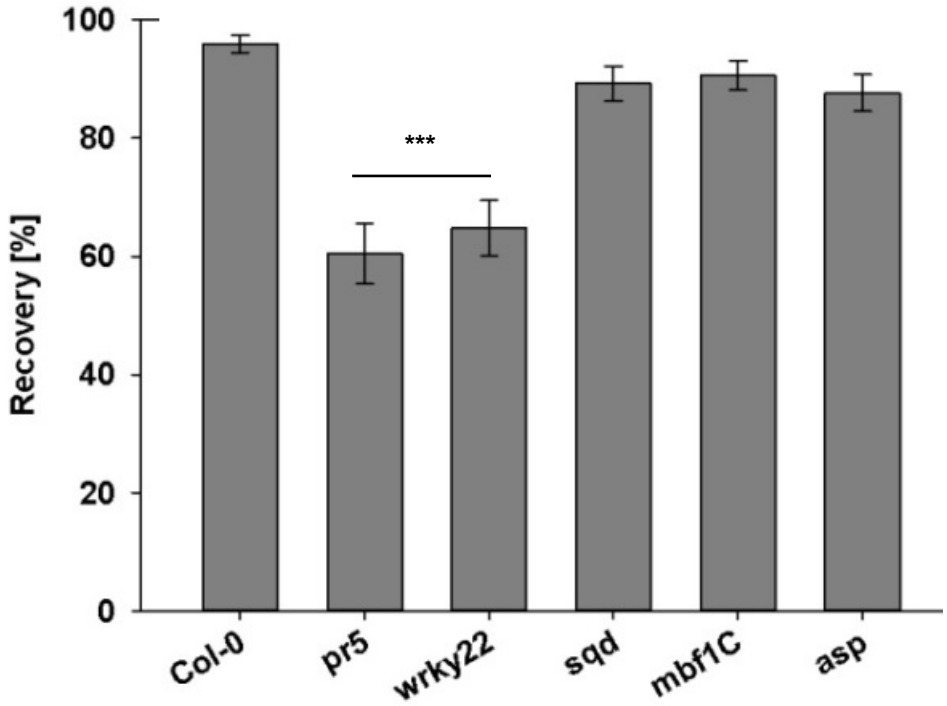



**Figure 9. Regeneration of *Arabidopsis* mutant plantlets after cryopreservation.** Percentage of recovered plantlets of Col-0 (WT), *pr5*: *PATHOGENESIS RELATED GENE 5*, *wrky22*: *WRKY22* transcription factor, *sqd1: SULFOQUINOVOSYLDIACYL GLYCEROL 1, mbf1C*: *MULTIPROTEIN BRIDGING FACTOR 1 C*, *asp: putative ASPARTYL PROTEASE.* The performance of the mutants was compared to that of WT using the Win Fisher test (***: $P \leq 0.001$; $n \geq 90$).

fully functional copy of either *WRKY22* or *PR5* resulted in a significantly impaired level of regeneration.

Since the absence of a functional copy of the transcripts results in a compromised level of post-cryogenic recovery, the genes *WRKY22* and *PR5* were of particular interest and further characterized towards the particular role during the multiple stress response of cryopreservation.

### 3.2.1. The role of *WRKY22* during cryopreservation

#### 3.2.1.1. The inactivation of *WRKY22* compromised the regrowth of cryopreserved explants

With respect to *WRKY22,* confirmation that the loss-of-function of the gene was responsible for the observed loss in regeneration was obtained by testing two independent T-DNA insertion mutants (*wrky22.1* and *wrky22.2*). The genes' highly conserved WRKY domain sequence was disrupted, since the T-DNA insertion was located in either the first or the third exon (**Figure 10**).

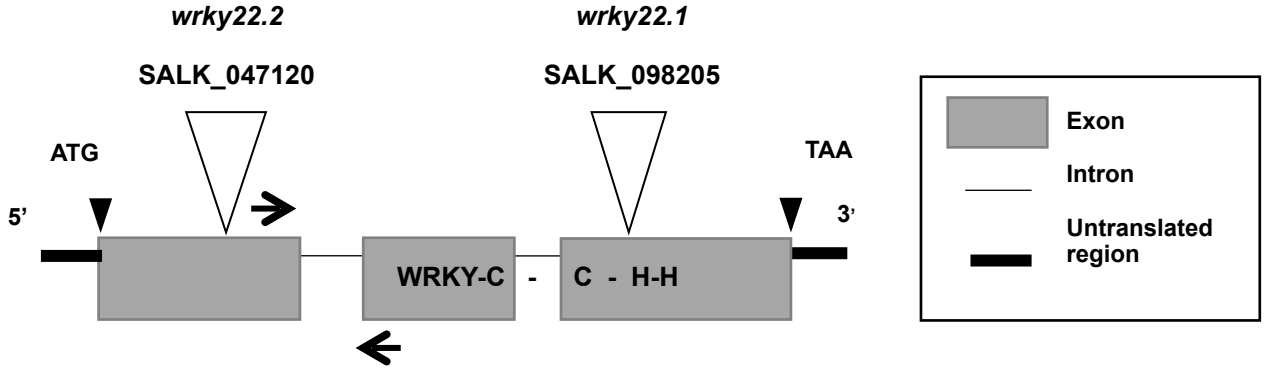



**Figure 10. Schematic T-DNA insertion sites in *WRKY22*.** For *wrky22.1* (SALK_098205), the T-DNA insertion was located in the third exon and for *wrky22.2* (SALK_047120) in the first exon. The highly conserved amino acid sequence of the WRKY domain [WRKYGQK sequence at the N-terminus and a zinc-finger-like motif Cys (2)-His (2) at the C-terminus] was located in the second and third exon. The location of the WRKY domain is indicated as WRKY-C-C-H-H. Arrows show primer combination for qRT-PCR.

The regeneration was reduced from 98 % for WT explants to 60 % for those derived from each of the KO mutants (**Figure 11A**) which showed reduced abundance of *WRKY22* transcript (**Figure 11B**). Introducing a copy of WT *WRKY22* driven by the CaMV 35S promoter (*35S:comp*) into the *wrky22.1* mutant both restored the level of *WRKY22* transcript to that measured in the WT explant and rescued the WT phenotype (**Figure 11A,B**).

The phenotypic appearance of WT, *wrky22* knockout mutants, and complementation plants (*35S:comp*) was further investigated (**Figure 11C-J**). Recovered plantlets from WT (**Figure 11C,D**) and *35S:comp* (**Figure 11E,F**) resembled one another with respect to their rosette leaves, roots and shoots, while the *wrky22* plantlets exhibited a distinct phenotype: 60 % of the plantlets retained a WT phenotype (**Figure 11G,I**), while 40 % developed incomplete leaves and roots, produced some callus material or stayed green without any further development, so called survival plantlets (**Figure 11H,J**)

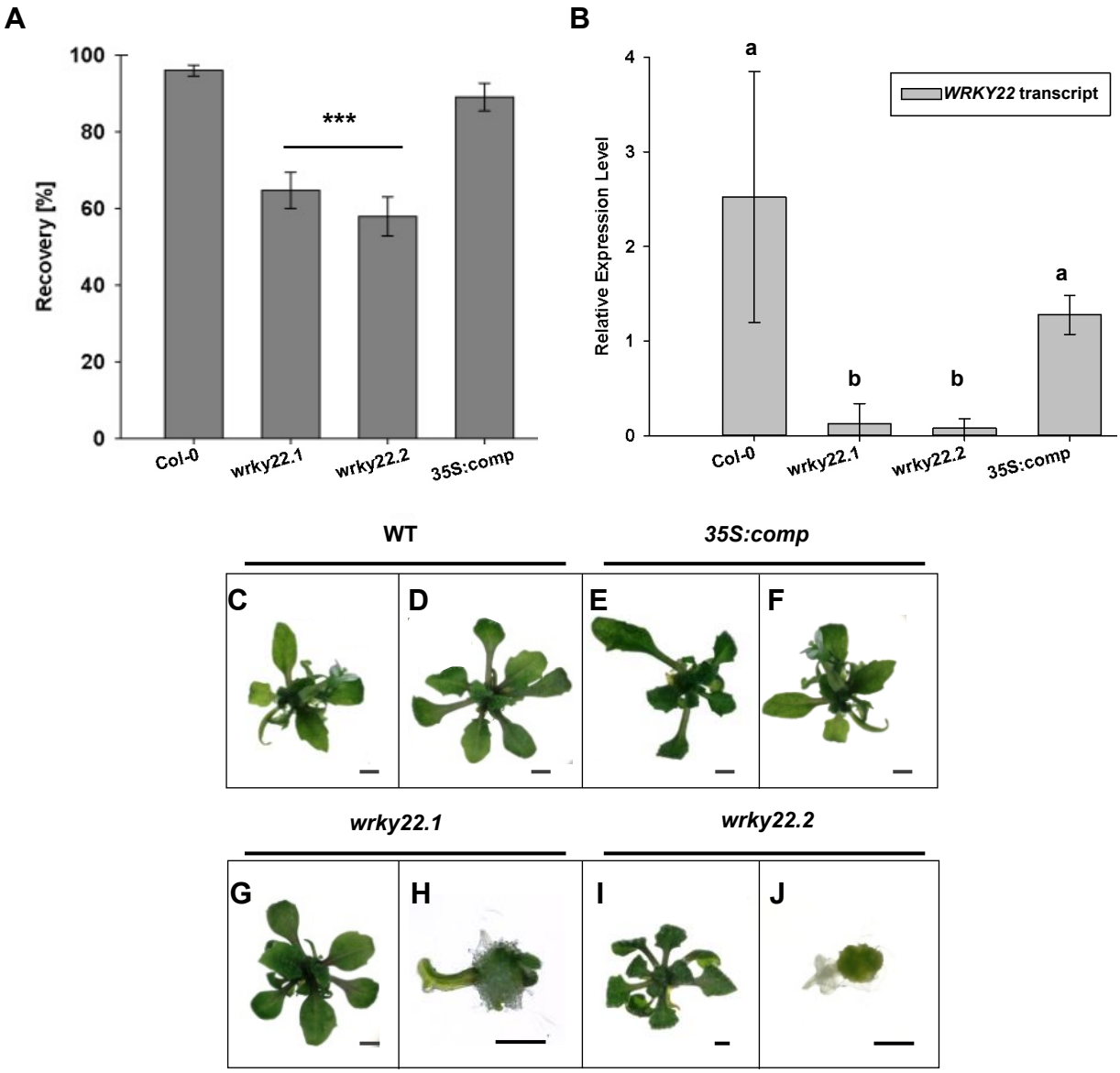



**Figure 11. Verification of the inactivated *WRKY22* transcript using *wrky22.1* and *wrky22.2* mutants and a transgenic *wrky22.1* mutant plant harboring the *35S:comp*.** (**A**) Recovery after cryopreservation comparing mutants to WT using the Win Fisher test (***: $P \leq 0.001$; $n \geq 90$). **(B)** Quantification of *WRKY22* transcript by qRT-PCR. Data were normalized to *TIP41 and CLATH*. Error bars indicate ±SD (n=3). Statistical significance was calculated using one-way ANOVA followed by Holm-Sidak post hoc test. Mean values marked by the same letter did not differ significantly from one another ($P \leq 0.05$). (**C-J**) The appearance of recovered plantlets derived from shoot tips of (**C,D**) WT, (E,F) transgenic *wrky22.1* mutant plant harboring the *35S:comp*, (**G,H**) *wrky22.1* mutant, (**I,J**) *wrky22.2* mutant. Scale bar: 1 mm.

### 3.2.1.2. *WRKY22* is induced during the second phase of cryopreservation

Quantitative reverse transcription PCR (qRT-PCR) of WT explants revealed that *WRKY22* transcription increased significantly over phase II, and decreased after the fourth day of phase III (**Figure 12**), indicating that *WRKY22* is likely involved in the response to PVS2-induced stress and the early phase of regeneration.

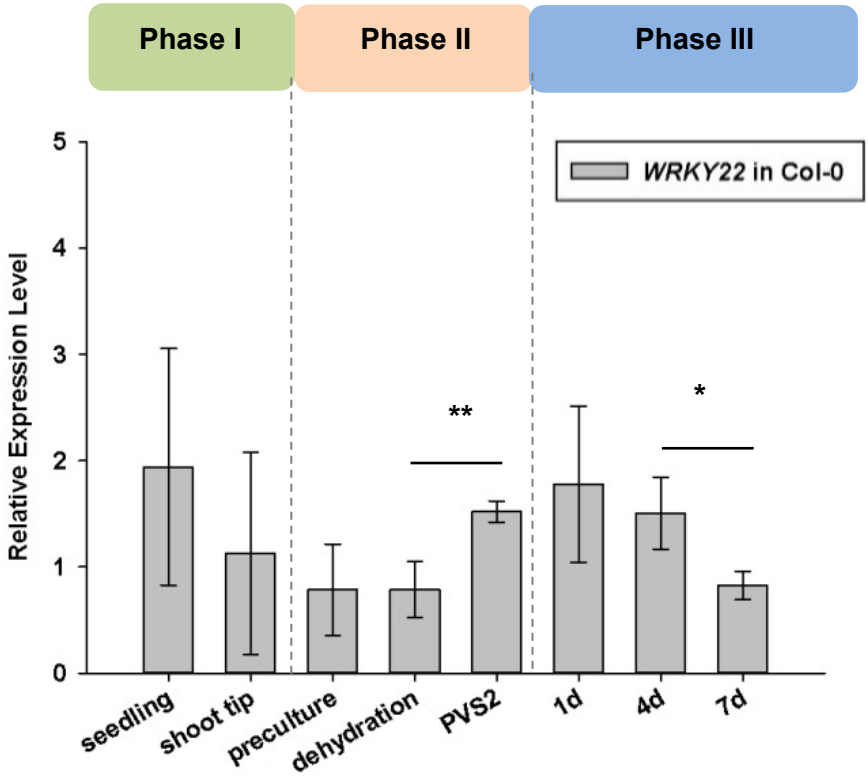



**Figure 12. Relative abundance of *WRKY22* transcript present in Col-0 (WT) explants sampled at each stages of the cryopreservation/recovery process.** The error bars indicate the SD (*n*=4). **, *: means differ at *P* ≤0.01 and ≤0.05, respectively, using one-way ANOVA followed by Holm-Sidak post hoc test.

### 3.2.1.3. PVS2 treatment is associated with ultrastructural changes to meristematic cells

When cross-sections of the first three layers of the explants' shoot apical meristem and the leaf primordia sampled during phase I were examined, both nuclei and vacuoles were prominent in the WT materials, and their cytoplasm appeared homogeneous and even structured (**Figure 13A,D**); the effect of the PVS2 treatment (phase II) was to reduce cell size, with the single large vacuole becoming replaced by several smaller ones (**Figure 13B**). The cells' organelles either increased in size or began to degrade, resulting in the formation of plastoglobuli (**Figure 13E**). During the early portion of phase III, the explants' ultrastructure resembled that seen during phase I: in particular, the symptoms associated with dehydration and preplastid degradation disappeared (**Figure 13C,F**). The ultrastructure of the *wrky22* mutant's shoot tip cells was indistinguishable from that of the WT cells (**Figure 13G-L**).

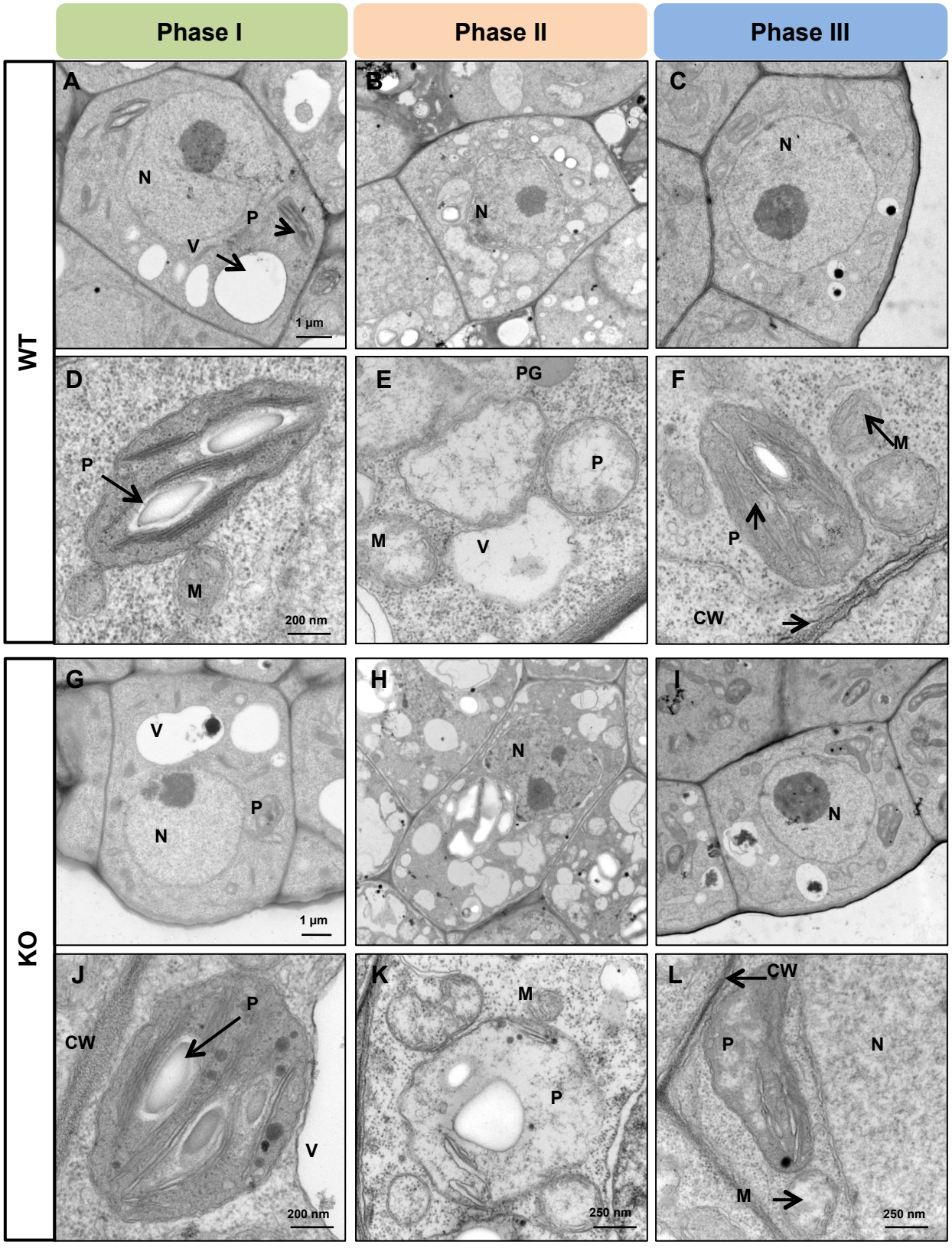



**Figure 13. Ultrastructural changes in cells of the apical shoot meristem in WT and KO.** Electron micrographs of meristematic cells after phase I, phase II, and phase III for (**A-F**) WT and (**G-L**) KO. CW: cell wall; M: mitochondrion; N: nucleus; P: preplastid; PG: plastoglobuli; St: starch; V: vacuole. Phase I: Shoot tip preparation; Phase II: PVS2 treatment; Phase III: First day of recovery. Transmission electron microscopy was done by Dr. Michael Melzer (IPK, Gatersleben).

### 3.2.1.4. The impact of cryostressors on the shoot tip's transcriptome

Changes in the WT shoot tip's transcriptome after shoot tip preparation (phase I), after PVS2 treatment (phase II), and after the first day of recovery (phase III) were analyzed. An additional comparison was made between the transcriptomes of the *wrky22.1* mutant and WT explants. The set of differentially expressed genes (DEGs) identified from these phases were then analyzed in the context of gene ontology (GO) term enrichment and MapMan-based clustering, and the 50 genes showing the highest and lowest $\log_2$ fold change were identified.

#### 3.2.1.4.1. The impact of the cryoprotectant treatment on RNA processing

The comparison between the WT explants' transcriptomes (shoot tips were sampled in phase I, II and III) identified 12,067 DEGs between phase I and II (**Figure 14A**), and between phases II and III 6,349 DEGs (**Figure 14B**).

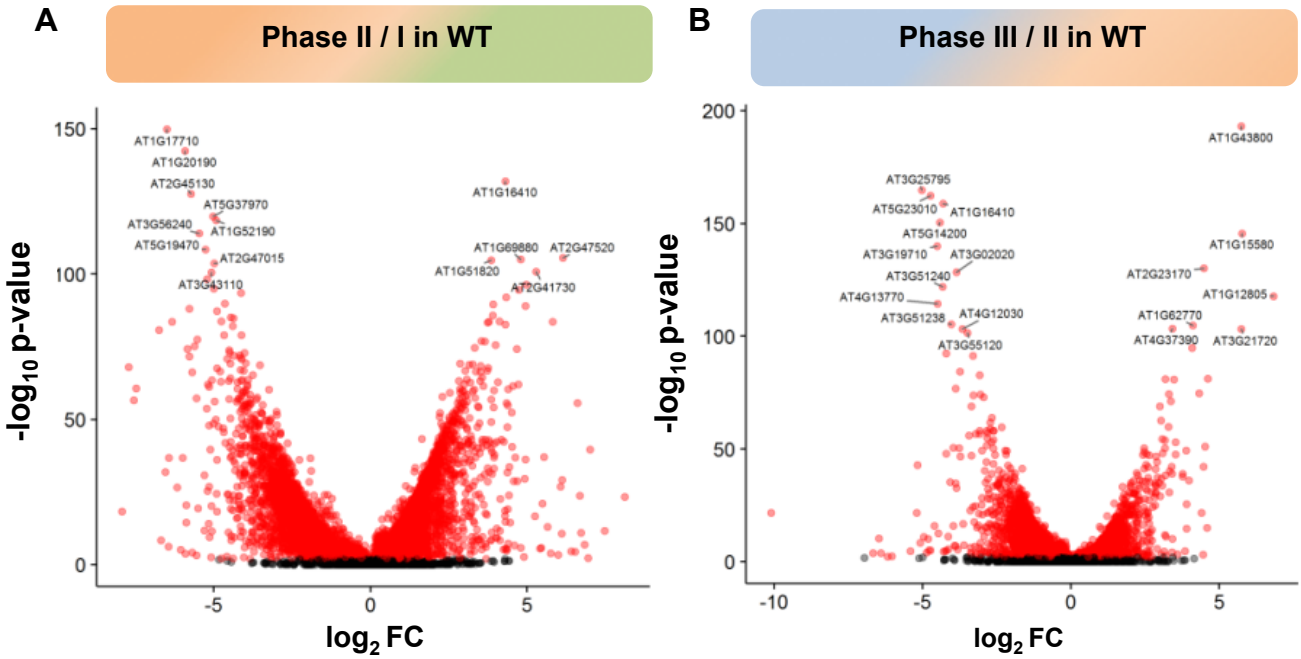



**Figure 14. Differential expressed genes of WT shoot tip explants at the end of phases I through III.** (**A**) The analysis identified 12,067 genes as changed with respect to their transcript abundance between phases I and II, and (**B**) 6,349 between phases II and III (p-value < 0.01 after multiple hypothesis correction). Genes associated with a p value $<10^{-100}$ are labeled with their AGI code. Phase I: Shoot tip preparation; Phase II: PVS2 treatment; Phase III: First day of recovery. Analysis was done by Prof. Andrea Bräutigam (Bielefeld University).

The two sets of DEGs (Phase II/I and III/I in WT) were assigned to MapMan bins, as depicted in **Figure 15A**. Most of the DEGs grouped either into bins related to primary metabolism (group 1), RNA processing/regulation (group 7), or protein synthesis/ modification (group 8).

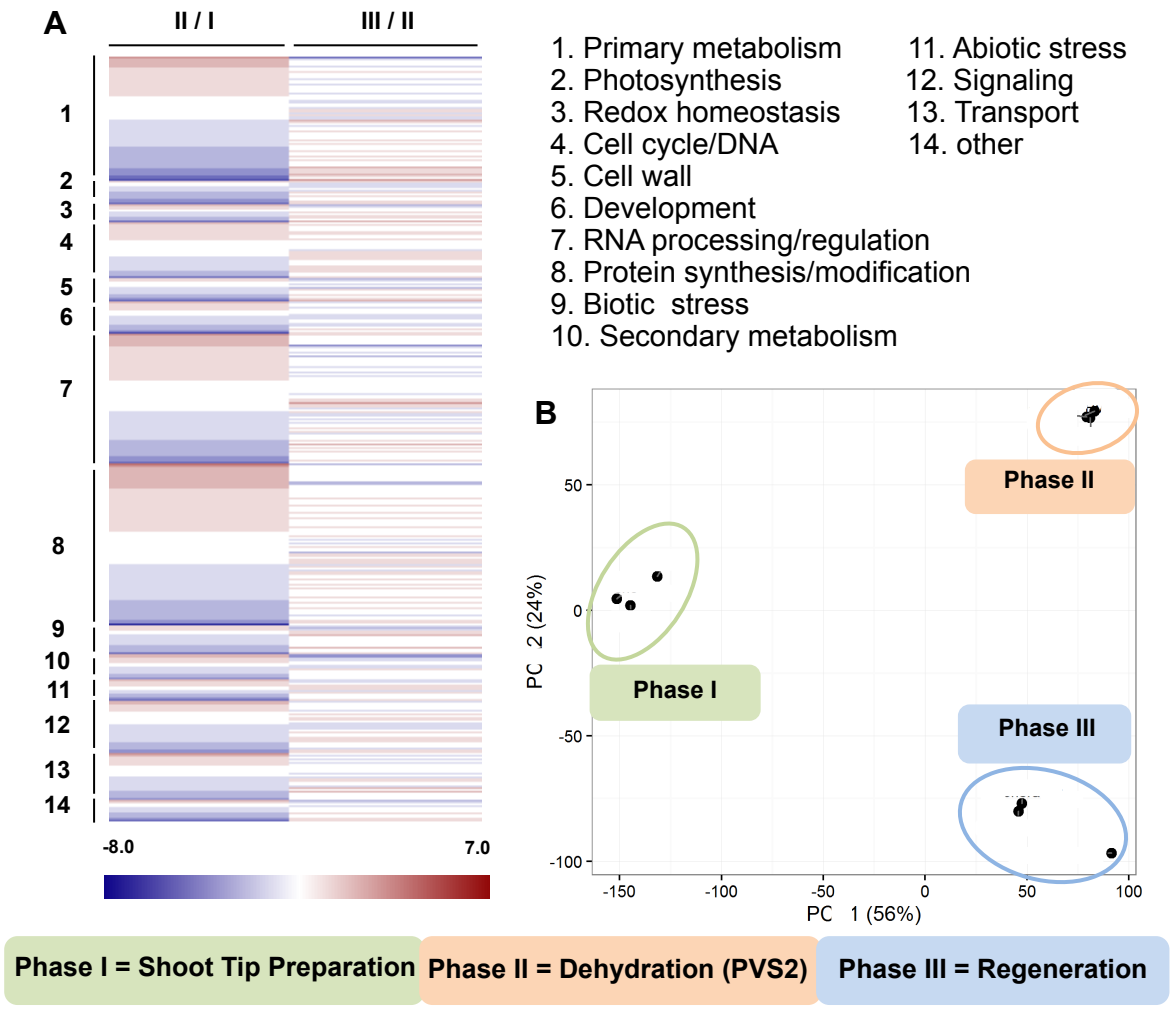



**Figure 15. The transcriptome of WT shoot tip explants at the end of phases I through III.** (**A**) The MapMan bins of the DEGs identified in the contrasts phases II *vs* I and III *vs* II. Red indicates increased abundance and blue decreased abundance, with the color intensity reflecting the fold of differential gene expression. (**B**) A principal component analysis confirms the difference between the three phases. Phase I: Shoot tip preparation; Phase II: PVS2 treatment; Phase III: First day of recovery. PCA was done by Prof. Andrea Bräutigam (Bielefeld University).

However, several DEGs could be clustered to terms of photosynthesis, redox homeostasis, cell cycle/DNA, cell wall, development, biotic stress, secondary metabolism, abiotic stress, signaling, transport, and other minor subgroups. The variance in the data set was classified by a principal component analysis (PCA). PC1 (representing 56% variance) and PC2 (representing 24% variance) showed a clear separation between the three phases (**Figure 15B**).

The GO enrichment analysis established that the PVS2 treatment had a major positive impact on the abundance of transcript generated by genes encoding proteins involved in RNA processing and methylation, mitochondrial processes, DNA modification and nuclear targeting. The major classes of genes negatively impacted were related to photosynthesis (in particular the light response and chlorophyll

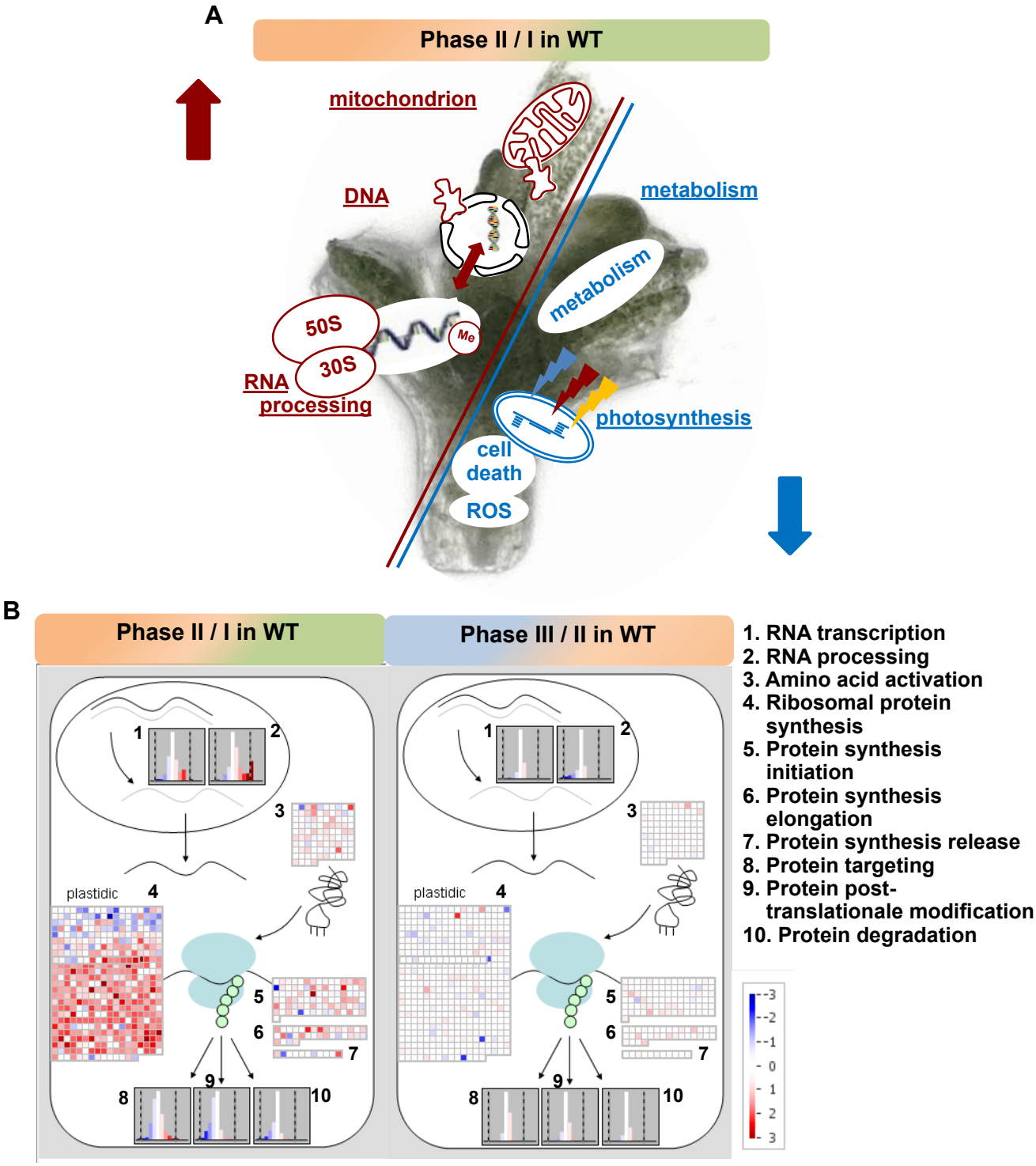



**Figure 16. GO term enrichment and MapMan functional assignment of the WT transcriptome in phase II *vs* I.** Enriched GO terms among the regulated genes in the contrast (**A**) phase II *vs* I and. Enriched GO-terms among genes with higher abundance are shown in red and among lower abundance in blue. (**B**) MapMan mapping of RNA-protein synthesis. Each square represents the transcription of a single gene within a given pathway. Hochberg-corrected transcripts with higher abundance are shown in red, and lower abundance in blue. The color intensity reflects the fold of differential gene expression. Phase I: Shoot tip preparation; Phase II: PVS2 treatment; Phase III: First day of recovery.

synthesis), as well as the metabolism of saccharides, lipids, fatty acids and amino acids (**Figure 16A**, **Appendix: Data Set 1**).

## RESULTS

The MapMan analysis confirmed the conclusions drawn from the GO term enrichment analysis: transcripts encoding proteins involved in ribosomal protein synthesis became notably more abundant in phase II than in phase I, but this difference was not apparent between phases II and III (**Figure 16B**, **Appendix Table 2**). Genes encoding components of RNA processing and ribosomal protein synthesis, as well as metabolism and photosynthesis were represented in the set of highest up- and down-regulated genes (**Tables 7, 8**, **Appendix: Data Set 2**). The transcriptomic data-based conclusions were validated for three selected RNA processing genes *NOP-56 like* (*At3G12860*; encodes for pre RNA processing ribonucleoprotein), *NOP-58 like* (*At3g05060*; a scaffold associated region (SAR) DNA-binding protein), and *EBP2* (*At3g22660*; encodes a rRNA processing protein-like protein involved in the biogenesis of the ribosomal large subunit), using qRT-PCR. In each case, transcript abundance was significant boosted by the PVS2 treatment compared to control seedlings and prepared shoot tips. After the first day of regeneration the relative expression level was depressed, but still at higher levels compared to control (**Figure 17**).

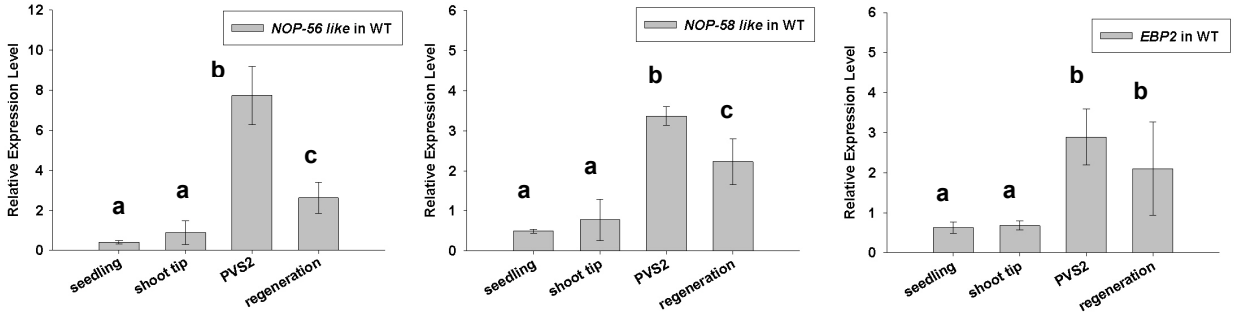



**Figure 17. Relative transcript expression of *NOP-56 like*, *NOP-58 like*, and *EBP2* in six weeks old seedlings and among different stages of cryopreservation (phase I, phase II, and phase III) in WT.** Data were normalized to *TIP41 and CLATH*. Error bars indicate SD (n=4). Mean values marked by the same letter did not differ significantly from one another ($P \leq 0.05$) analyzed with one-way ANOVA followed by Holm-Sidak post-hoc.

Beside the identified genes important for RNA processing, five genes known to be inducible by drought stress (*HRE2* (*At4g06746; HYPOXIA RESPONSIVE ETHYLENE RESPONSE FACTOR 2*), *RAP2.9* (*At2g47520*), *DR4* (*At1g73330; DROUGHT REPRESSED 4*), *ERD10* (*At1g20450; EARLY RESPONSIVE TO DEHYDRATION*) and *CBF1* (*At4g25490; C-REPEAT/DRE BINDING FACTOR 1*), along with two low temperature stress- inducible genes (*COR47* (*At1g20440*) and *COR413IM1* (*At1g29395*)) could be found in the hit lists of transcript with highest or lowest abundance after PVS2 treatment. These DEGs are marked by a single asterisk in

**Table 7. The 50 most highly up-regulated genes in the WT explants identified in the contrast phase II *vs* I.** MapMan bins consistent with GO term enrichment are shown in bold. Genes labeled with an asterisk have been associated in the literature with either the drought stress response (*) or products of secondary metabolism (**).

| | Locus | FC | Gene Name | MapMan Functional |
|---|---|---|---|---|
| **1*** | **AT2G47520** | **6.1** | ***HYPOXIA RESPONSIVE (ERF) 2 (HRE2)*** | **RNA.regulation of transcription** |
| 2 | AT4G12490 | 6.1 | *AZI3* | misc.protease inhibitor |
| 3 | AT3G46280 | 5.0 | *kinase-like protein* | signalling.receptor kinases |
| 4 | AT2G26150 | 4.9 | *HEAT SHOCK TRANSCRIPTION FACTOR A2* | stress.abiotic.heat |
| **5** | **AT1G10585** | **4.9** | ***basic helix-loop-helix DNA-binding superfamily*** | **RNA.regulation of transcription** |
| 6 | AT1G69880 | 4.8 | *THIOREDOXIN H-TYPE 8* | redox.thioredoxin |
| **7** | **AT5G59240** | **4.7** | ***Ribosomal protein S8e family protein*** | **protein.synthesis.ribosomal** |
| **8** | **AT3G17609** | **4.7** | *HY5-HOMOLOG* | **RNA.regulation of transcription** |
| 9 | AT4G22470 | 4.6 | *Protease inhibitor/lipid-transfer protein* | misc.protease inhibitor |
| **10** | **AT5G51440** | **4.5** | ***HSP20-like chaperones superfamily protein*** | **stress.abiotic.heat** |
| **11** | **AT1G05680** | **4.4** | ***UGT74E2*** | **hormone metabolism.salicylic** |
| **12*** | **AT4G06746** | **4.4** | ***RELATED TO AP2 9 (RAP2.9)*** | **RNA.regulation of transcription** |
| 13** | AT3G51240 | 4.4 | *FLAVANONE 3-HYDROXYLASE* | secondary metabolism.flavonoids |
| **14** | **AT1G64220** | **4.4** | ***TRANSLOCASE OF OUTER MEMBRANE 7-2*** | **transport mitochondrial** |
| 15 | AT1G17180 | 4.4 | *GLUTATHIONE S-TRANSFERASE TAU 25* | misc.glutathione S transferases |
| **16** | **AT3G09680** | **4.3** | ***Ribosomal protein S12/S23 family protein*** | **protein.synthesis.ribosomal** |
| 17** | AT1G16410 | 4.3 | *CYTOCHROME P450 79F* | secondary metabolism.sulfur- |
| 18 | AT2G16060 | 4.1 | *HEMOGLOBIN 1* | redox.heme |
| 19** | AT5G13930 | 4.1 | *CHALCONE SYNTHASE* | secondary metabolism.flavonoids |
| **20** | **AT5G14200** | **4.0** | *ISOPROPYLMALATE DEHYDROGENASE 1* | **amino acid metabolism.synthesis** |
| **21** | **AT5G40040** | **4.0** | ***60S acidic ribosomal protein family*** | **protein.synthesis.ribosomal** |
| 22 | AT5G39580 | 4.0 | *Peroxidase superfamily protein* | misc.peroxidases |
| 23 | AT2G15620 | 3.9 | *NITRITE REDUCTASE 1* | N-metabolism.nitrate metabolism |
| **24** | **AT3G19710** | **3.9** | *BRANCHED-CHAIN AMINOTRANSFERASE4* | **amino acid metabolism.synthesis** |
| **25** | **AT3G12860** | **3.9** | ***NOP56-like pre RNA processing*** | **protein.synthesis.ribosome** |
| 26 | AT5G41670 | 3.9 | *6-phosphogluconate dehydrogenase family protein* | OPP.oxidative |
| 27 | AT3G46230 | 3.9 | *HSP17.4* | stress.abiotic.heat |
| 28** | AT5G07990 | 3.9 | *CYTOCHROME P450 75B1* | secondary metabolism.flavonoids |
| **29** | **AT1G32880** | **3.9** | ***ARM repeat superfamily protein*** | **protein.targeting.nucleus** |
| 30 | AT1G51820 | 3.9 | *Leucine-rich repeat protein kinase family protein* | signalling.receptor kinases.misc |
| **31** | **AT1G58684** | **3.8** | ***Ribosomal protein S5 family protein*** | **protein.synthesis.ribosomal** |
| **32** | **AT1G58983** | **3.8** | ***Ribosomal protein S5 family protein*** | **protein.synthesis.ribosomal** |
| 33 | AT1G02820 | 3.8 | *LEA3* | development |
| **34** | **AT3G06900** | **3.8** | ***U4 SMALL NUCLEOLAR RNA2*** | **RNA.processing** |
| 35 | AT5G40850 | 3.8 | *UROPHORPHYRIN METHYLASE 1* | tetrapyrrole synthesis |
| 36 | AT1G51850 | 3.8 | *Leucine-rich repeat protein kinase family protein* | signalling.receptor kinases.misc |
| 37 | AT4G33070 | 3.7 | *ATPDC1* | fermentation.PDC |
| 38 | AT1G24280 | 3.7 | *G6PD3* | OPP.oxidative |
| 39** | AT5G23010 | 3.7 | *2-ISOPROPYLMALATE SYNTHASE 3* | secondary metabolism.sulfur- |
| 40 | AT4G12480 | 3.7 | *PEARLI 1* | misc.protease inhibitor |
| **41** | **AT1G23410** | **3.7** | ***Ribosomal protein S27a*** | **protein.synthesis.ribosomal** |
| 42 | AT4G12500 | 3.6 | *Bifunctional inhibitor/lipid-transfer protein* | misc.protease inhibitor |
| 43 | AT1G78050 | 3.6 | *PGM* | glycolysis.unclear |
| 44 | AT1G14120 | 3.6 | *AUXIN OXIDASE* | misc.oxidases |
| **45** | **AT5G13490** | **3.6** | ***ADP/ATP CARRIER 2*** | **transport.unspecified cations** |
| **46** | **AT3G02020** | **3.5** | ***ASPARTATE KINASE 3*** | **amino acid metabolism** |
| 47 | AT2G03230 | 3.5 | *GCK domain-containing protein* | not assigned.unknown |
| **48** | **AT4G25630** | **3.5** | ***FIBRILLARIN 2*** | **protein.synthesis.ribosome** |
| **49** | **AT5G27120** | **3.5** | ***NOP58-like pre RNA processing*** | **RNA.regulation of transcription** |
| **50** | **AT5G53290** | **3.5** | ***CYTOKININ RESPONSE FACTOR 3*** | **RNA.regulation of transcription** |

**Table 8. The 50 most highly down-regulated genes in the WT explants identified in the contrast phase II *vs* I.** MapMan bins consistent with GO term enrichment are shown in bold. Genes labeled with an asterisk have been associated in the literature with either the drought stress response (*) or products of secondary metabolism (**).

| | Locus | FC | Gene Name | Mapman Functional Description |
|---|---|---|---|---|
| **1** | **AT2G33830** | **-7.7** | ***DORMANCY ASSOCIATED GENE 2*** | **hormone metabolism** |
| 2 | AT1G31580 | -7.6 | *ECS1* | stress.biotic |
| **3** | **AT1G56600** | **-7.5** | ***GALACTINOL SYNTHASE 2*** | **minor CHO metabolism** |
| 4* | AT1G20440 | -6.5 | *COLD-REGULATED 47* | stress.abiotic.unspecified |
| 5 | AT1G17710 | -6.5 | *Pyridoxal phosphate phosphatase-related* | misc.acid and other phosphatases |
| 6 | AT1G26945 | -6.4 | *PACLOBUTRAZOL RESISTANCE 6* | not assigned.unknown |
| 7 | AT5G45890 | -6.2 | *SENESCENCE-ASSOCIATED GENE 12* | protein.degradation |
| 8 | AT1G20190 | -5.9 | *EXPANSIN 11* | cell wall.modification |
| 9* | AT1G29395 | -5.9 | *COLD REGULATED 314 INNER MEMBRANE 1* | not assigned.no ontology |
| 10 | AT1G52690 | -5.9 | *LATE EMBRYOGENESIS ABUNDANT* | development |
| 11 | AT3G09922 | -5.8 | *INDUCED BY PHOSPHATE STARVATION1* | not assigned.unknown |
| 12 | AT1G56220 | -5.8 | *Dormancy/auxin associated family protein* | development.unspecified |
| 13 | AT2G45130 | -5.7 | *SPX DOMAIN GENE 3* | stress.abiotic |
| 14* | AT1G73330 | -5.7 | *DROUGHT-REPRESSED 4* | stress.biotic |
| **15** | **AT3G27690** | **-5.6** | ***LHCB2.3*** | **PS.lightreaction.photosystem II** |
| 16 | AT5G24490 | -5.6 | *30S ribosomal protein* | protein.synthesis.ribosomal protein |
| **17** | **AT3G01500** | **-5.5** | ***SALICYLIC ACID-BINDING PROTEIN 3*** | **TCA / org transformation** |
| **18** | **AT3G15450** | **-5.5** | ***Aluminium induced protein*** | **hormone metabolism** |
| 19 | AT3G56240 | -5.5 | *COPPER CHAPERONE* | metal handling |
| 20 | AT5G14565 | -5.3 | *MICRORNA398C* | micro RNA, natural antisense |
| **21** | **AT1G09350** | **-5.3** | ***GALACTINOL SYNTHASE 3*** | **minor CHO metabolism** |
| **22** | **AT5G19470** | **-5.3** | ***NUDIX HYDROLASE HOMOLOG 24*** | **nucleotide metabolism** |
| **23** | **AT3G26180** | **-5.2** | ***CYP71B20*** | **misc.cytochrome P450** |
| **24** | **AT3G02040** | **-5.2** | ***SENESCENCE-RELATED GENE 3*** | **lipid metabolism** |
| 25* | AT1G20450 | -5.2 | *EARLY RESPONSIVE TO DEHYDRATION 10* | stress.abiotic.unspecified |
| 26 | AT2G41870 | -5.2 | *Remorin family protein* | RNA.regulation of transcription |
| 27 | AT3G16670 | -5.2 | *Pollen Ole e 1 allergen* | not assigned.unknown |
| **28** | **AT3G26740** | **-5.2** | ***CCR-LIKE*** | **signalling.light** |
| **29** | **AT1G80920** | **-5.2** | ***TOC12*** | **stress.abiotic.heat** |
| 30 | AT5G06760 | -5.1 | *LATE EMBRYOGENESIS ABUNDANT 4-5* | development |
| **31** | **AT3G62550** | **-5.1** | ***Adenine nucleotide alpha hydrolases-like protein*** | **hormone metabolism** |
| 32 | AT3G55240 | -5.1 | *Protein coding* | not assigned.unknown |
| 33 | AT5G37970 | -5.0 | *S-adenosyl-L-methionine-dependent* | **hormone metabolism.salicylic** |
| 34* | AT4G25490 | -5.0 | *DRE BINDING PROTEIN 1B (CBF1)* | RNA.regulation of transcription |
| **35** | **AT3G63210** | **-5.0** | ***MEDIATOR OF ABA-REGULATED DORMANCY 1*** | **hormone metabolism** |
| 36 | AT2G47015 | -5.0 | *MICRORNA408* | micro RNA, natural antisense |
| 37 | AT1G75380 | -5.0 | *BIFUNCTIONAL NUCLEASE IN BASAL DEFENSE* | stress.abiotic.touch/wounding |
| 38 | AT1G67265 | -4.9 | *ROTUNDIFOLIA LIKE 21* | development.unspecified |
| 39 | AT1G52190 | -4.9 | *NITRATE TRANSPORTER 1.11* | transport.peptides and oligopeptides |
| 40 | AT5G49360 | -4.9 | *BETA-XYLOSIDASE 1* | cell wall.degradation |
| 41 | AT1G01470 | -4.9 | *LATE EMBRYOGENESIS ABUNDANT 14* | development |
| **42** | **AT1G23730** | **-4.9** | ***BETA CARBONIC ANHYDRASE 3*** | **TCA / org transformation** |
| **43** | **AT1G79040** | **-4.9** | ***PHOTOSYSTEM II SUBUNIT R*** | **PS.lightreaction.photosystem II** |
| 44 | AT1G18870 | -4.9 | *ISOCHORISMATE SYNTHASE 2* | Co-factor and vitamine metabolism |
| 45 | AT2G17040 | -4.9 | *NAC DOMAIN CONTAINING PROTEIN 36* | development.unspecified |
| **46** | **AT1G20620** | **-4.8** | ***CATALASE 3*** | **redox.dismutases and catalases** |
| 47 | AT1G73540 | -4.8 | *NUDIX HYDROLASE HOMOLOG 21* | nucleotide metabolism |
| 48 | AT5G39520 | -4.8 | *hypothetical protein* | not assigned.unknown |
| **49** | **AT1G11530** | **-4.7** | ***ATCXXS1*** | **redox.thioredoxin** |
| 50 | AT1G28330 | -4.7 | *DORMANCY-ASSOCIATED PROTEIN 1* | development.unspecified |

**Tables 7 and 8** and indicated a certain importance of osmotic adaptation towards cryoprotectant treatment. Further, the set of genes with higher abundance included a number of transcripts associated with the defense response, in particular related to products of secondary metabolism (marked with a double asterisk in **Table 7**).

A GO term enrichment analysis of the set of DEGs in phase III indicated that genes with higher abundance could be assigned to terms of development, cell cycling, protein modification/ubiquitination and DNA modification/replication, while apoptosis and defense were suppressed (**Figure 18A**, **Appendix: Data Set 1**).

Consistent with MapMan analysis, transcripts showing lower abundance were prominently related to products of secondary metabolism, especially transcript related to lignin, flavonoids, anthocyanins, and glucosinolates (**Figure 18B**). Candidate genes representing reduced defense capacity were *MAM1* (*At5G23010*; *METHYLTHIO-ALKYLMALATE SYNTHASE 1*), *F3H* (*At3G51240*; *FLAVANONE 3-HYDROXYLASE*), and genes grouping to the *CHALCONE-FLAVANONE ISOMERASE PROTEIN FAMILY* (*TRANSPARENT TESTA 4,5,7*). The ones with higher abundance belonged to the group of auxin-mediated cell growth like *ATAUX2-27* (*At1G15580*), *AUXIN RESISTANT 5* (*At4G14560*), and *IAA19* (*At3G15540*) (**Figure 18C**). Genes were also liste in the set of highest up or down regulated genes in **Appendix Data Set 2**.

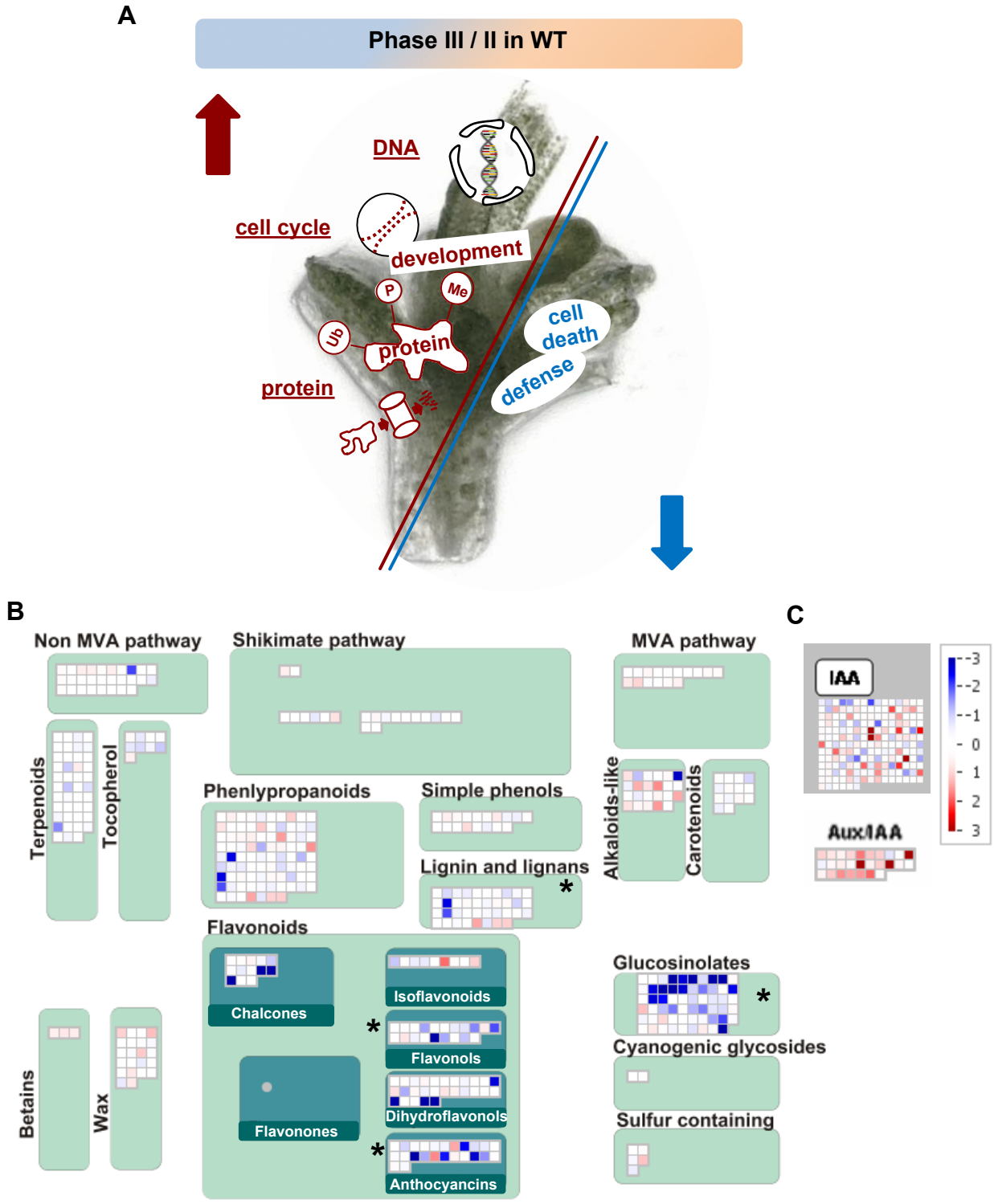



**Figure 18. GO term enrichment and MapMan functional assignment of the WT transcriptome in phase III *vs* II.** Enriched GO terms among the regulated genes in the contrast (**A**) phase III *vs* II. Enriched GO-terms among genes with higher abundance are shown in red and among lower abundance in blue. MapMan mapping of (**B**) secondary metabolism and (**C**) auxin/IAA transcript regulation in the contrast phase III *vs* II in WT. Each square represents the transcription of a single gene within a given pathway. Hochberg-corrected transcripts with higher abundance are shown in red, and lower abundance in blue. The color intensity reflects the fold of differential gene expression. *: *P*<0.05 after Benjamini Hochberg correction. Phase II: PVS2 treatment; Phase III: First day of recovery.

### 3.2.1.4.2. The transcription of genes involved in photosynthesis is affected by exposure to the cryoprotectant treatment in the *wrky22.1* mutant shoot tip explants

In all, 124 genes were differentially transcribed between WT and the *wrky22* mutant when the explants were sampled at the end of phase I (**Figure 19A**), as were 2,599 (**Figure 19B**) at the end of phase II and 1,119 at the end of phase III (**Figure 19C**). Lower transcript abundance of *ARABIDOPSIS MTO 1 RESPONDING DOWN 1* (*MRD1*) (*At1G53480*) could be detected in *wrky22.1* KO shoot tips compared to WT among all three phases.

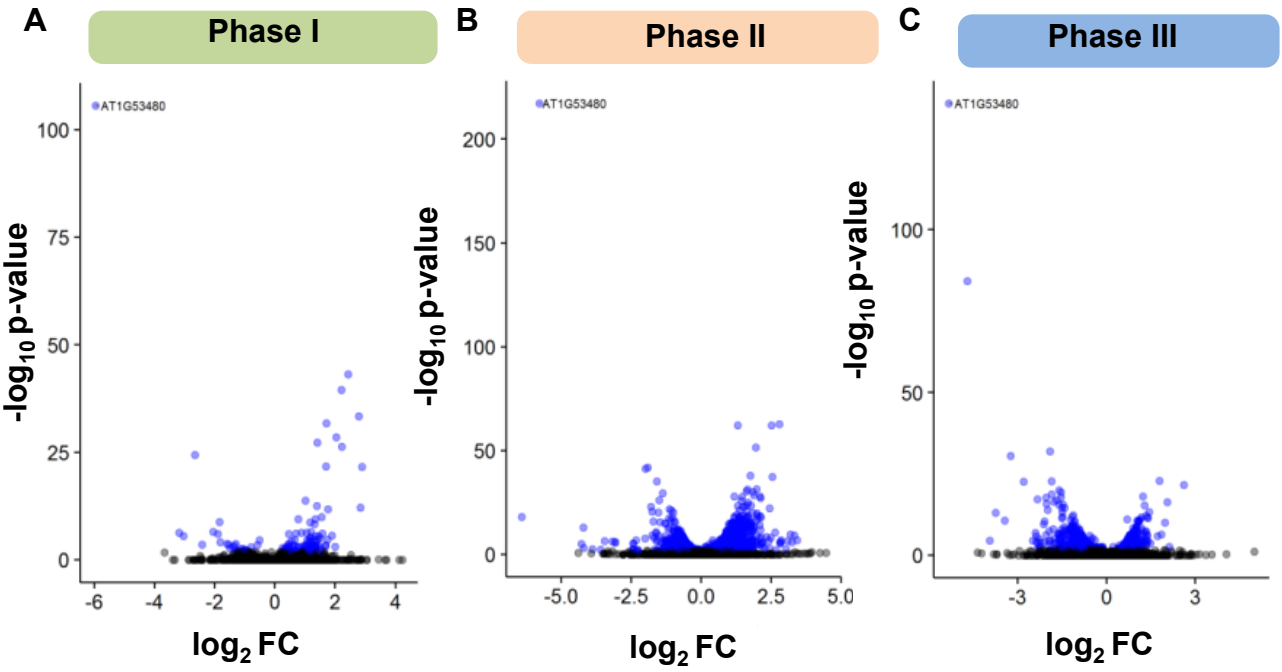



**Figure 19. The transcriptome of *wrky22.1* mutant shoot tip explants at the end of phase I through III.** The analysis identified **(A)** 124 genes as changed with respect to their transcript abundance in phase I, **(B)** 2,599 genes in phase II, and **(C)** 1,119 genes in phase III (p-value < 0.01 after multiple hypothesis correction). Genes associated with a p value $<10^{-100}$ are labeled with their AGI code. Phase I: Shoot tip preparation; Phase II: PVS2 treatment; Phase III: First day of recovery. Analysis was done by Prof. Andrea Bräutigam (Bielefeld University).

A principal component analysis clearly distinguished the three phases, and highlighted the genotypic difference between the WT and the *wrky22* mutant in phases II and III (**Figure 20**).

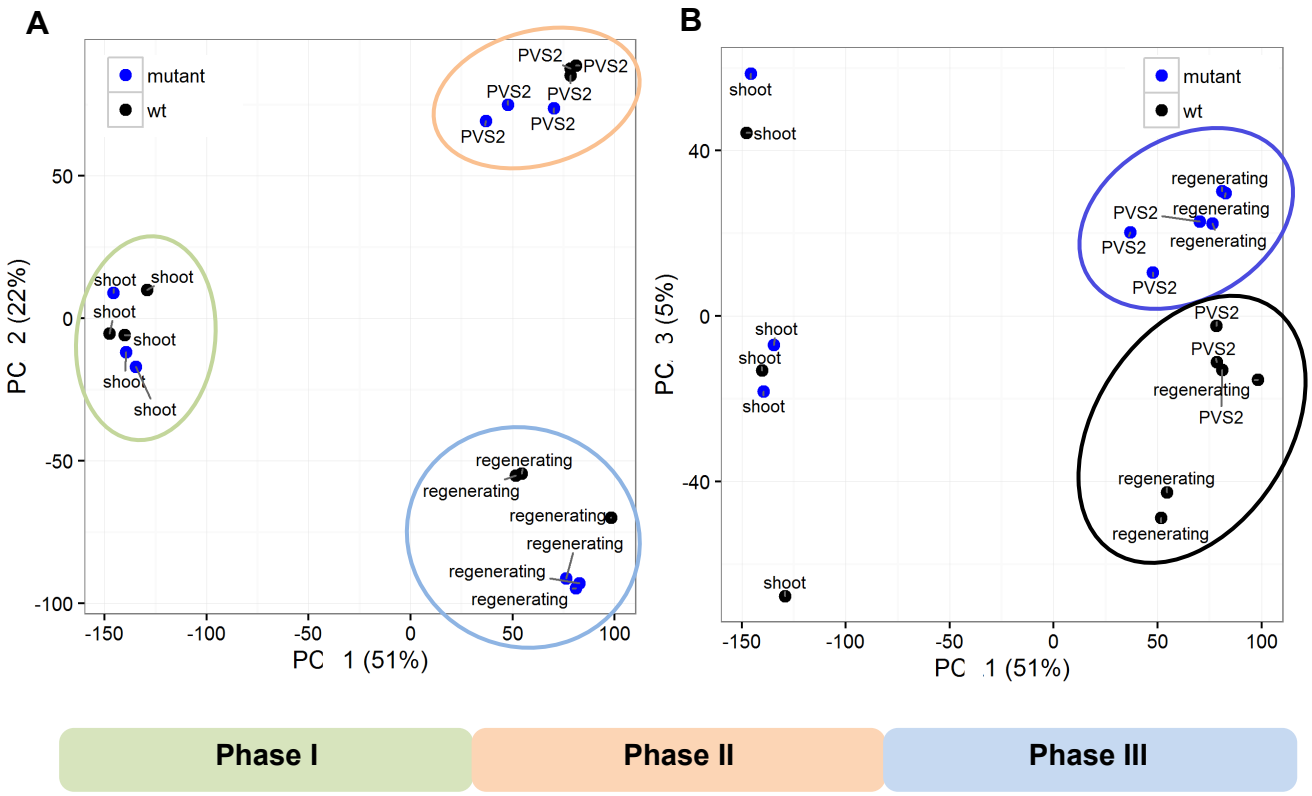
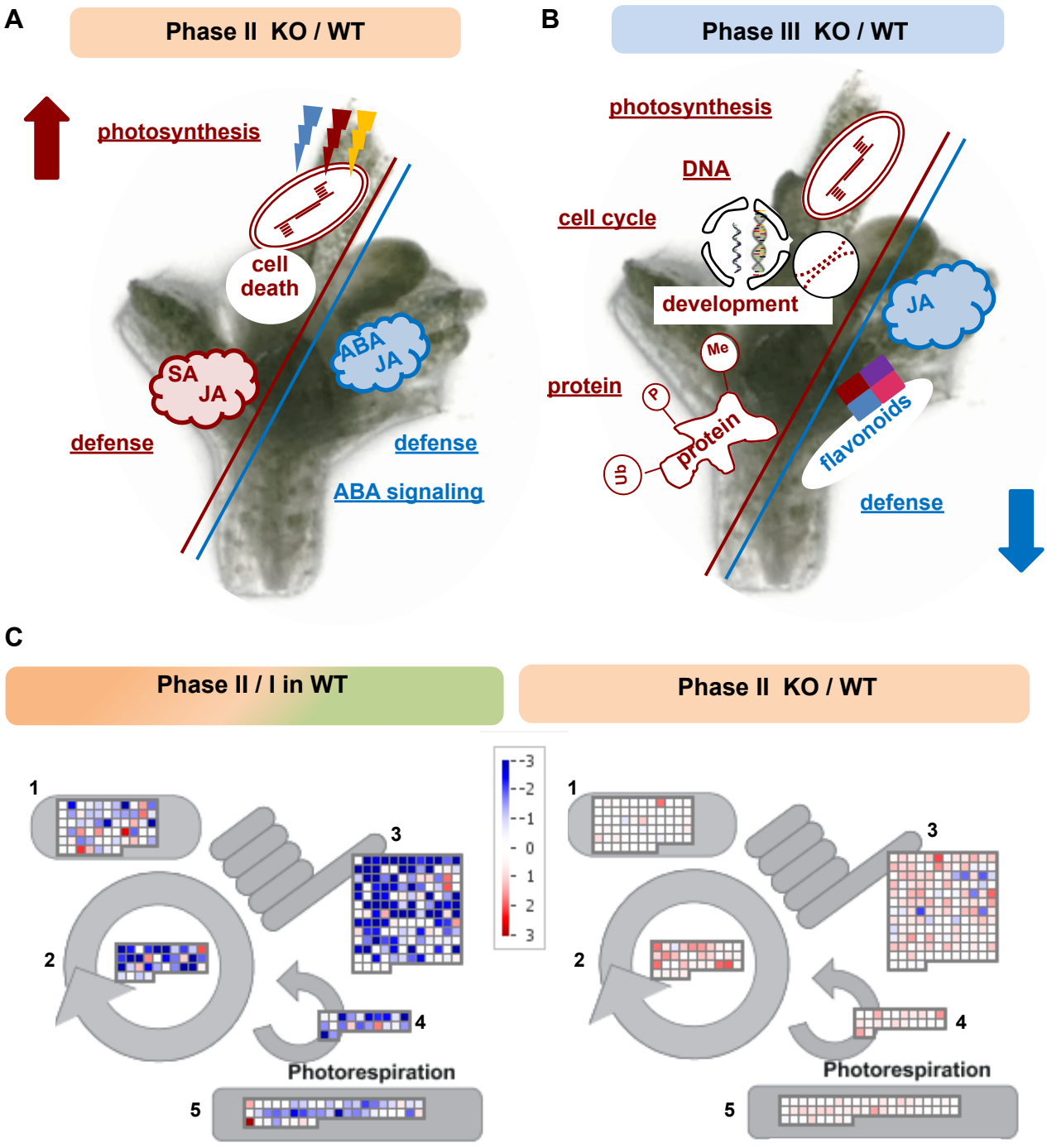



**Figure 20. A principal component analysis confirms the separation of the transcriptome of *wrky22.1* mutant shoot tip explants at the end of phase I through III** in the first two dimensions according to (**A**) treatment and **(B)** genotypic effects in the third dimension. Analysis was done by Prof. Andrea Bräutigam (Bielefeld University).

Effects of *WRKY22* were marginal during shoot tip preparation. The most over-represented category of DEGs was associated with secondary cell wall synthesis, in particular genes encoding for *PROLINE-RICH EXTENSIN-LIKE FAMILY PROTEINS* (**Appendix: Data Set 1, Data Set 2**). These findings suggested that the *wrky22* mutant shoot tips were compromised with respect to the strength of their secondary cell walls.

A GO term enrichment analysis of the phase II DEG set showed promoted GO terms in the *wrky22* mutant explants related to photosynthesis, the response to light, reactive oxygen species homeostasis and apoptosis. Genes encoding the SA- and jasmonic acid (JA)-regulated defense response, along with products of secondary metabolism were over-represented in both the high- and low-abundance categories, while processes relying on abscisic acid (ABA) signaling were suppressed (**Figure 21A**, **Appendix: Data Set 1**).

During phase III, development, DNA/RNA modification, protein modification and photosynthesis were all promoted in the *wrky22* mutant, while the JA-mediated defense response and flavonoid synthesis were suppressed (**Figure 21B**, **Appendix: Data Set 1**).




**1. Redox ascorbate and glutathione; 2. Calvin cycle; 3. PS lightreaction; 4. PS photorespiration; 5. Tetrapyrrole synthesis**

**Figure 21. GO term enrichment and MapMan functional assignment of the *wrky22.1* mutant transcriptome.** Enriched GO terms among the regulated genes in the contrast (**A**) phase II and (**B**) phase III in *wrky22.1* mutant over WT. Enriched GO-terms among genes with higher abundance are shown in red and among lower abundance in blue. (**C**) MapMan mapping of primary metabolism. Each square represents the transcription of a single gene within a given pathway. Hochberg-corrected transcripts with higher abundance are shown in red, and lower abundance in blue. The color intensity reflects the fold of differential gene expression. Phase I: Shoot tip preparation; Phase II: PVS2 treatment; Phase III: First day of recovery. PS: photosystem.

The MapMan analysis confirmed the outcome of the GO term enrichment analysis with respect to genes involved in photosynthesis during phase II. In particular, genes related to redox homeostasis (1. Redox ascorbate and glutathione), Calvin Cycle (2.), photosynthesis light reaction (3.) and photorespiration (4.), as well as tetrapyrrole synthesis (5.), showed reduced abundance in WT shoot tips at phase II compared to mutant levels (**Figure 21C**).

Three of these genes, namely *RCA* (*At2g39730*; *RUBISCO ACTIVASE* involved in the regulation of the Calvin Cycle), *PSAN* (*At5g64040*; encodes the only subunit of photosystem I located entirely in the thylakoid lumen), and *RBCS3B* (*At5g38410*; encodes *RUBISCO SMALL SUBUNIT 3B*), were chosen for qRT-PCR validation purposes. The analysis showed that their transcript abundance was lower during phase II than during phase I in both the *wrky22* mutant and the WT explants, and that it was higher in the *wrky22* mutant than in WT during phase II (**Figure 22**).

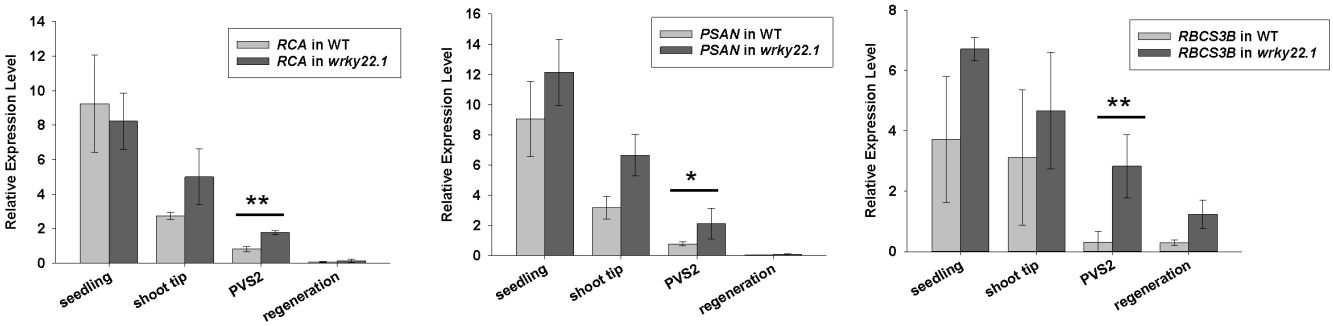



**Figure 22. Relative transcript expression of *RCA*, *PSAN*, and *RBCS3B* in six weeks old seedlings and among different stages of cryopreservation (phase I, phase II, and phase III) in WT and *wrky22.1* mutant.** Data were normalized to *TIP41 and CLATH*. Error bars indicate +SD (n=4). Statistical differences between WT and *wrky22.1* mutant after PVS2 treatment were calculated using student t-test (* $P \leq 0.05$; ** $P \leq 0.01$).

### 3.2.1.4.3. *WRKY22* is involved in phytohormone-mediated drought and defense adaptation through cross-talk with assorted transcription factors

The set of DEGs (selected on the basis of a $\log_2$ fold change threshold in transcript abundance of 1.5) between phases I and II for WT, and between the *wrky22.1* mutant and WT during phase II, was assembled to identify potential targets of *WRKY22.* Of these, 145 were assigned to MapMan bins associated with development, hormone and transcript regulation, biotic stress and photosynthesis (**Figure 23A**). The group of DEGs assigned to the hormone and regulation category included a number of members of the AP2-ERF, MYB and WRKY TF families. Four of these (*WRKY71* (*At1g29860*), *WIN1* (*At5g11190*), *WRKY53* (*At4g23810*) and *WRKY70* (*At3g56400*))

are known to be inducible by more than one stress agent. The products of certain dehydration-responsive binding protein/C-repeat binding factor encoding genes (*CBF*s), as well as those of *DDF 1* (*At1g12610*), *HRE2* (*At2g47520*) and *GAL-OXI* (*At3g27220*) are known to be involved in the regulation of the osmotic stress response, while those of *RAV2* (*At1g68840*), *TCL2* (*At2g30424*) and *RAD-LIKE3* (*At4g36570*) control two or more developmental processes; finally, the product of *ORA47* (*At1g74930*) has been identified as acting in the JA-regulated defense response (see references **Figure 23B**). The presence of *WRKY22* resulted in the suppression of most of the genes (the exceptions were *GAL-OXI* and *HRE2*), which supported the existence of cross-talk during osmotic stress adaptation between *WRKY22* and members of the AP2-ERF, MYB and WRKY families. Overall, 25 putative interaction partners of *WRKY22* in response to osmotic stress were identified (**Table 9**).

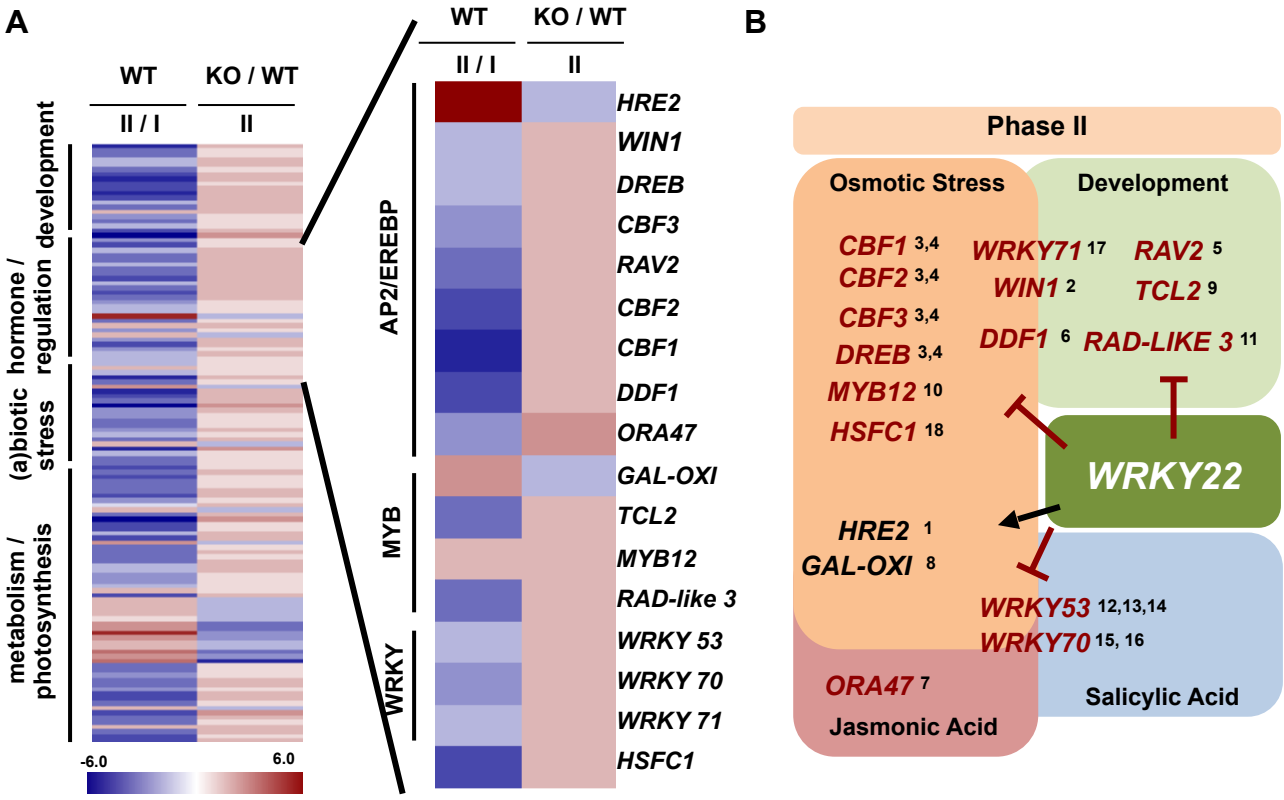



**Figure 23. Cross-talk between transcription factors associated with *WRKY22* activity.** (**A**) A heat map identifying putative interaction partners with *WRKY22* during phase II belonging to the AP2-ERF, MYB and WRKY transcription factor family. Genes differentially expressed between phases II and I in WT explants were chosen on the basis of a $\log_2$ fold change of >1.5, and in the *wrky22.1* mutant explants on the basis of a $\log_2$ fold change >1.3. Red indicates increased abundance and blue decreased abundance, with the color intensity reflecting the fold of differential gene expression. (**B**) Assignment of function in the context of the biotic and/or abiotic stress response. Genes repressed by *WRK22* indicated by a red arrow, and those promoted by it by a black arrow. [1]Park et al., 2011, [2] Al-Abdallat et al., 2014, [3] Novillo et al., 2004, [4] Sakuma et al., 2002, [5] Matías-Hernández et al., 2016, [6] Kang et al., 2011, [7] Chen et al., 2016, [8] Loreti et al., 2005, [9] Tominaga-Wada et al., 2013, [10] Wang et al., 2016, [11] Baxter et al., 2007, [12] Sun et al., 2003, [13] Sun and Yu, 2015, [14] Miao and Zentgraf, 2007, [15] Li et al., 2013, [16] Chen et al., 2017 [17] Guo and Quin, 2016, [18] Rizhsky et al., 2004.

**Table 9. DEGs identified in explants from the contrast phase II *vs* I in WT (on the basis of a $log_2$ fold change threshold of 1.5) and in phase II *wrky22.1* mutant *vs* WT (on the basis of a $log_2$ fold change threshold of 1.3)**. Gene functions assigned either by MapMan analysis or from the literature in context of the regulation of osmotic stress.

| | Locus | WT II / I | KO II | Description | Mapman Functional Description | Reference |
|---|---|---|---|---|---|---|
| 1 | AT1G01470 | -4.9 | 1.4 | *LATE EMBRYOGENESIS ABUNDANT 14 (LEA14)* | development.late embryogenesis abundant | Jia et al. 2014 |
| 2 | AT1G12610 | -3.9 | 1.7 | *DWARF AND DELAYED FLOWERING 1 (DDF1)* | RNA.regulation of transcription.AP2/EREBP | Kang et al., 2011; Magome et al., 2008 |
| 3 | AT4G25490 | -5.0 | 1.7 | *C-repeat/DRE binding factor 1 (CBF1)* | RNA.regulation of transcription.AP2/EREBP | Liu et al., 1998; Sakuma et al., 2002 |
| 4 | AT4G25470 | -4.3 | 1.7 | *C-repeat/DRE binding factor 2 (CBF2)* | RNA.regulation of transcription.AP2/EREBP | Liu et al., 1998; Sakuma et al., 2002 |
| 5 | AT4G25480 | -2.1 | 1.4 | *C-repeat/DRE binding factor 2 (CBF3)* | RNA.regulation of transcription.AP2/EREBP | Liu et al., 1998; Sakuma et al., 2002 |
| 6 | AT5G21960 | -1.6 | 1.4 | *DREB* | RNA.regulation of transcription.AP2/EREBP | Liu et al., 1998; Sakuma et al., 2002 |
| 7 | AT2G47520 | 6.1 | -1.9 | *HYPOXIA RESPONSIVE ETHYLENE RESPONSE FACTOR 2 (HRE2)* | RNA.regulation of transcription.AP2/EREBP | Park et al., 2011 |
| 8 | AT2G47460 | 1.8 | 1.8 | *MYB12* | RNA.regulation of transcription.MYB | Wang et al., 2016 |
| 9 | AT3G27220 | 2.4 | -1.8 | *Galactose oxidase/kelch repeat superfamily (GAL-OXI)* | RNA.regulation of transcription.MYB | Loreti et al 2005 |
| 10 | AT3G56400 | -2.5 | 1.3 | *WRKY70* | RNA.regulation of transcription.WRKY | Li et al., 2013; Chen et al., 2017 |
| 11 | AT4G23810 | -2.0 | 1.3 | *WRKY53* | RNA.regulation of transcription.WRKY | Sun et al., 2003; Sun and Yu, 2015; |
| 12 | AT1G29860 | -1.9 | 1.6 | *WRKY71* | RNA.regulation of transcription.WRKY | Guo and Quin, 2016 |
| 13 | AT4G11650 | -4.2 | 1.3 | *OSMOTIN 34 (OSM34)* | stress.abiotic | Sharma et al., 2013 |
| 14 | AT3G24520 | -3.8 | 1.4 | *Heat shock transcription factor C1 (HSFC1)* | RNA.regulation.transcription.HSF | Rizhsky et al 2004 |
| 15 | AT1G20440 | -6.5 | 2.2 | *COLD REGULATED 47 (COR47)* | stress.abiotic.unspecified | Wu et al., 2017 |
| 16 | AT1G20450 | -5.1 | 1.6 | *EARLY RESPONSIVE TO DEHYDRATION 10 (ERD10)* | stress.abiotic.unspecified | Wu et al., 2017 |
| 17 | AT5G66400 | -4,5 | 1.6 | *RESPONSIVE TO ABA 18 (RAB18)* | stress.abiotic.unspecified | Wu et al., 2017 |
| 18 | AT1G73330 | -5.6 | 2.8 | *DROUGHT-REPRESSED 4 (DR4)* | stress.biotic.PR-proteins.proteinase inhibitors | Boyce et al., 2003 |
| 19 | AT3G62410 | -4.0 | 1.5 | *CP12 domain-containing protein 2 (CP12-2)* | PS.calvin cycle | López-Calcagno et al., 2017 |
| 20 | AT3G54050 | -3.7 | 1.5 | *HIGH CYCLIC ELECTRON FLOW 1 (HCEF1)* | PS.calvin cycle.FBPase | Soto-Suárez et al., 2016 |
| 21 | AT1G32060 | -4.0 | 1.5 | *PHOSPHORIBULOKINASE (PRK)* | PS.calvin cycle.PRK | López-Calcagno et al., 2017 |
| 22 | AT2G39730 | -4.0 | 1.6 | *RUBISCO ACTIVASE (RCA)* | PS.calvin cycle.rubisco interacting | Zhang et al., 2015b |
| 23 | AT1G29395 | -5.8 | 1.6 | *COLD REGULATED 314 INNER MEMBRANE 1 (COR413IM1)* | not assigned.no ontology | Magome et al., 2008 |
| 24 | AT1G62480 | -4.0 | 1.3 | *Vacuolar calcium-binding protein-related* | signalling.calcium | Boyce et al., 2003 |
| 25 | AT4G17340 | -4.3 | 1.4 | *tonoplast intrinsic protein 2;2 (TIP2;2)* | transport.Major Intrinsic Proteins.TIP | Zhu et al., 2014 |

### 3.2.1.5. Stomatal aperture induced by PVS2 treatment differed between WT and the *wrky22* mutants

To reveal the function of *WRKY22* in stomatal movement, leaves of both the WT and the two independent *wrky22* mutants were treated with either an opening solution (OS), ABA or PVS2 (**Figure 24**). The treatment with the opening solution induced opening of guard cells for all three genotypes. Both treatments, ABA and PVS2, promoted the closure of guard cells in the WT leaf (**Figure 24A**) but not in those of either mutant (**Figure 24B,C**). In **Figure 24D** stomatal closure dependent on treatment is visualized. These results implied that the loss of *WRKY22* function induced a greater level of sensitivity to osmotic stress.

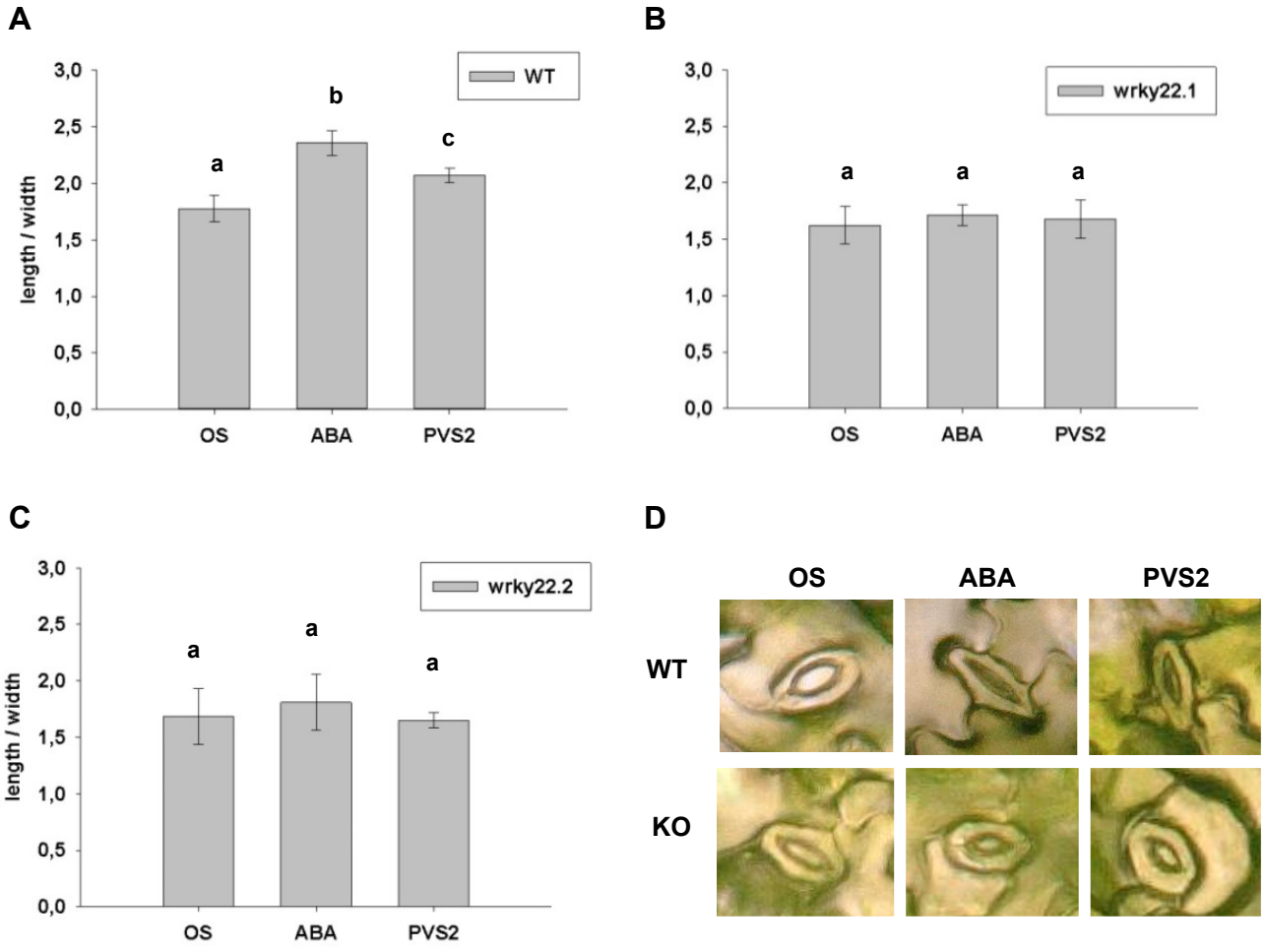



**Figure 24. Changes in stomatal aperture induced by *WRKY22* in the presence of opening solution (OS), ABA, and PVS2.** (**A**) WT, (**B**) *wrky22.1* mutant, (**C**) *wrky22.2* mutant. (**D**) Visual confirmation of changes in stomatal aperture. The stomatal aperture ratio (length/ width) was calculated from 80 stomata in three biological replicates; SD (*n*=3). Statistical significance was calculated using one-way ANOVA followed by Holm-Sidak post hoc test. Mean values marked by the same letter did not differ significantly from one another ($P \leq 0.05$).

## RESULTS

A follow-up experiment, in which water was withheld from both WT and *wrky22* mutant plants for 18 days, confirmed that the loss of *WRKY22* function significantly affect viability parameter. Compared to WT, wrky22 knockout mutants showed reduced size of rosette (**Figure 25A**) and plants' fresh weight (**Figure 25B**). As a consequence, a clear phenotype was observed for drought stressed *wrky22* loss of function mutants (**Figure 25C**).

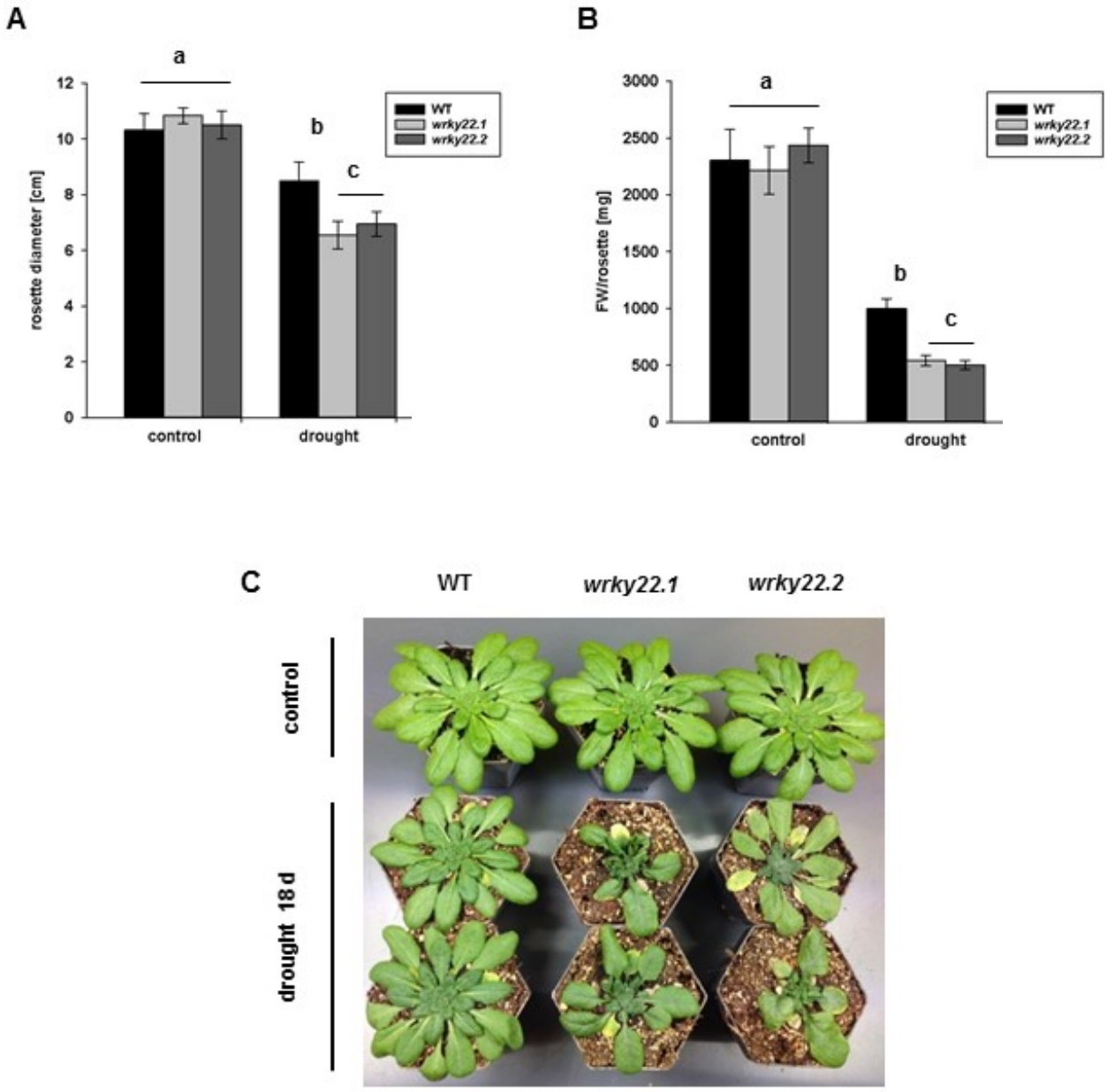



**Figure 25. Drought stress induced *wrky22* phenotype.** Loss of *WRKY22* function reduced (**A**) rosette diameter (**B**) fresh weight (FW) and results in a (**C**) drought stress sensitive phenotype after 18 d without watering (drought). Statistical significance was calculated using one-way ANOVA followed by Holm-Sidak post hoc test. Mean values marked by the same letter did not differ significantly from one another ($P \leq 0.05$).

### 3.2.2. The role of *PR5* during cryopreservation

#### 3.2.2.1. The inactivation of *PR5* compromised the regrowth of cryopreserved explants

The effect of knocking out the gene *At1g75040* (*PR5*) on the performance of cryopreserved explants was explored by exposing a T-DNA knock-out line (*pr5*) to the cryostorage protocol. Since the T-DNA insertion was located in the second exon, a functional copy of *PR5* was not expressed (**Figure 26**).

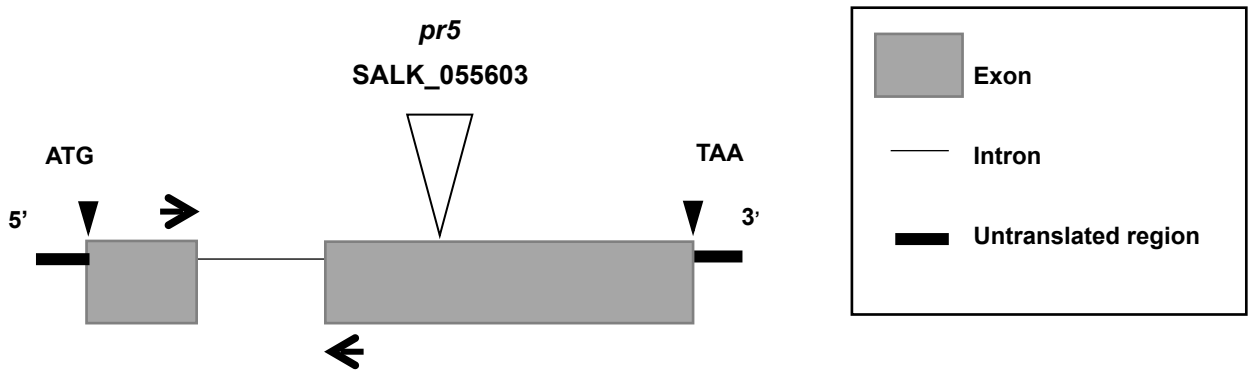



**Figure 26. Schematic T-DNA insertion sites in *PR5*.** For *pr5*, the T-DNA insertion was located in the second exon (SALK_055603). Arrows show primer combination for qRT-PCR.

The vast majority (98 %) of WT shoot tips regenerated into viable plantlets after cryogenic treatment. In contrast, the lack of a fully functional copy of *PR5* resulted in a significantly impaired level of regeneration. The regeneration rate was reduced from 98 % for WT explants to less than 60 % for those derived from the mutants (**Figure 27A**). Introducing a copy of WT *PR5* driven by the CaMV 35S promoter (*35S:comp*) into the *pr5* mutant both restored the level of *PR5* transcript to that measured in the WT explant and rescued the WT phenotype (**Figure 27A,B**).

Further, phenotypic appearance was evaluated for WT, *35S:comp*, and *pr5* loss of function mutant, 25 days after cryopreservation. Recovered plantlets from WT (**Figure 27C,D**) and *35S:comp* (**Figure 27E,F**) resembled one another with respect to their rosette leaves, roots and shoots. In contrast, the *pr5* mutant plantlets exhibited a distinct phenotype: 60% of the plantlets retained a WT phenotype (**Figure 27G**), while 40 % developed incomplete leaves and roots, produced some callus material or stayed green without any further development (**Figure 27H**).

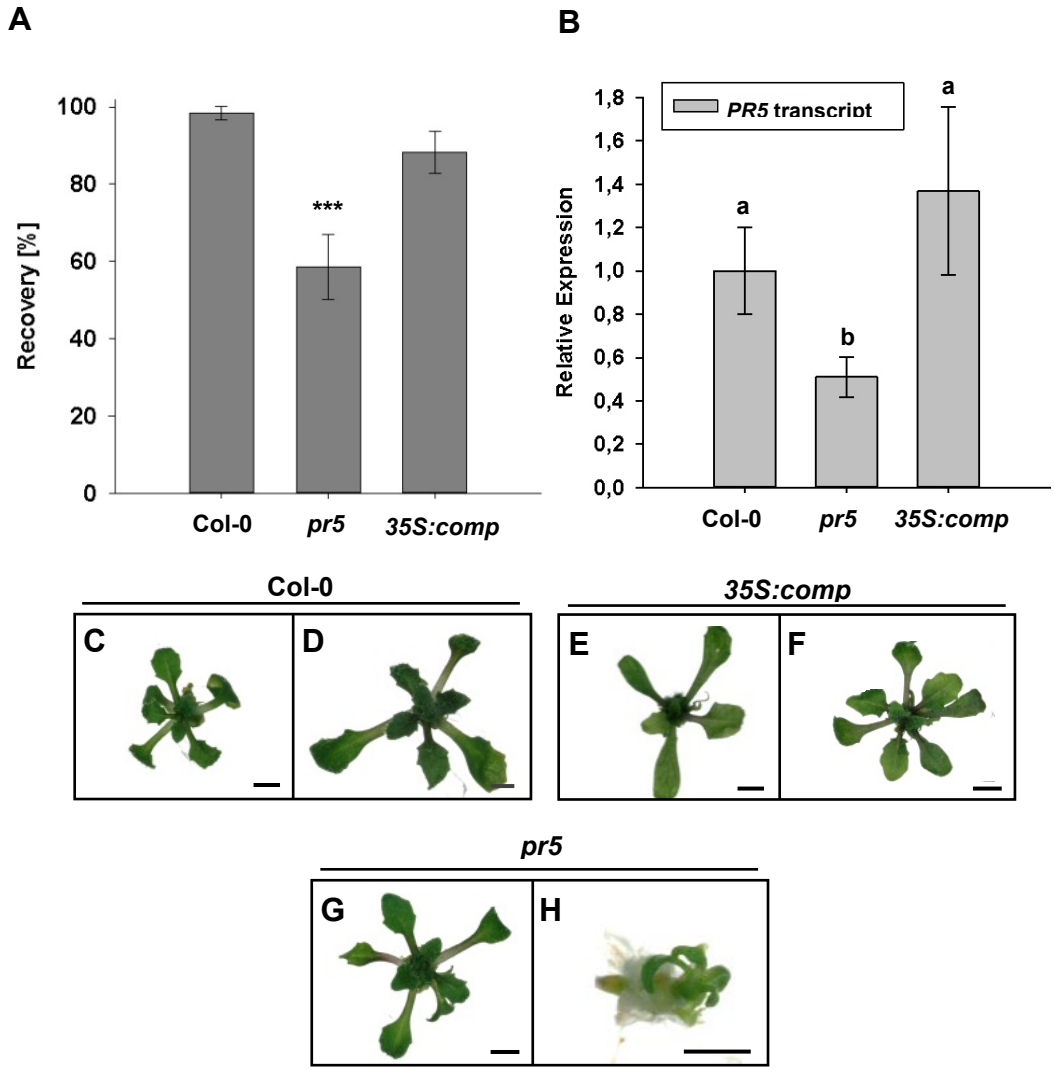



**Figure 27. Verification of the inactivated *PR5* transcript using *pr5* mutant and a transgenic *pr5* mutant plant harboring the *35S:comp*.** (**A**) Recovery after cryopreservation comparing mutants to WT using the Win Fisher test (***: $P \leq 0.001$; $n \geq 90$). **(B)** Quantification of *PR5* transcript by qRT-PCR. Data were normalized to *TIP41 and CLATH*. Error bars indicate ±SD (n=3). Statistical significance was calculated using one-way ANOVA followed by Holm-Sidak post hoc test. Mean values marked by the same letter did not differ significantly from one another ($P \leq 0.05$). (**C-J**) The appearance of recovered plantlets derived from shoot tips of (**C,D**) WT, (**E,F**) transgenic *pr5* mutant plant harboring the *35S:comp*, (**G,H**) *pr5* mutant. Scale bar: 1 mm.

### 3.2.2.2. Cellular *PR5* expression is induced during the second phase of cryopreservation

qRT-PCR of WT explants after different phases of the cryopreservation procedure revealed that *PR5* transcription increased significantly in phase II and decreased during phase III (**Figure 28**). These results suggested that *PR5* is likely involved in the response to wound-induced stress and the early phase of dehydration.

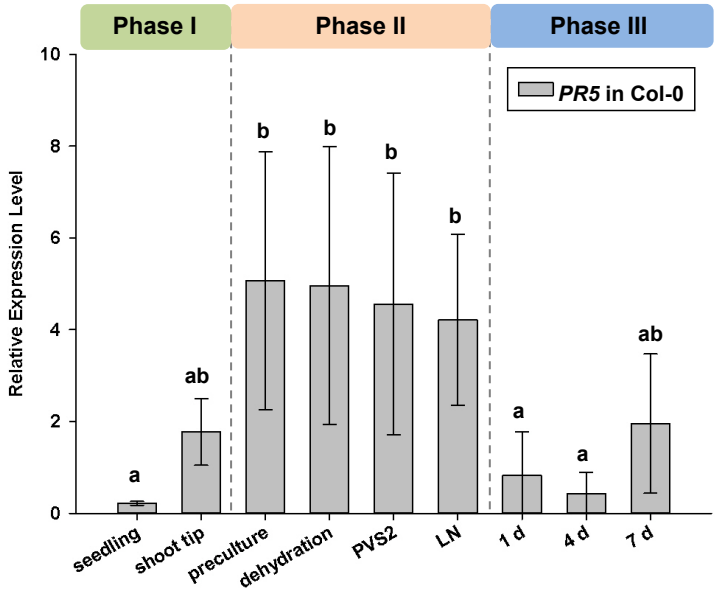



**Figure 28. Relative abundance of *PR5* transcript present in Col-0 (WT) explants sampled at each stages of the cryopreservation/recovery process.** The error bars indicate the SD (*n*=6). Mean values marked by the same letter did not differ significantly from one another (*P*≤0.05) analyzed with one-way ANOVA followed by Holm-Sidak post-hoc.

Subcellular localization of 35S:GFP-PR5-construct in *N. benthamiana* protoplasts showed a cytoplasm specific localization (**Figure 29A-C**). The RNA-*seq* dataset was further analyzed to prove that cytosolic *PR5* transcript expression is induced as a late response, dependent on the level of tissue wounding after shoot tip preparation and the accompanied oxidative burst. Data analysis elucidated 33 transcripts related to redox regulatory pathways with highest transcript abundance after shoot tip preparation, and more than 1.5 $log_2$FC reduced abundance or were even no expression during cryoprotectant treatment. Transcripts from this group mainly comprised enzymatic antioxidants like *CATALASE 3* (*At1g20620*) and *DEHYDROASCORBATE*

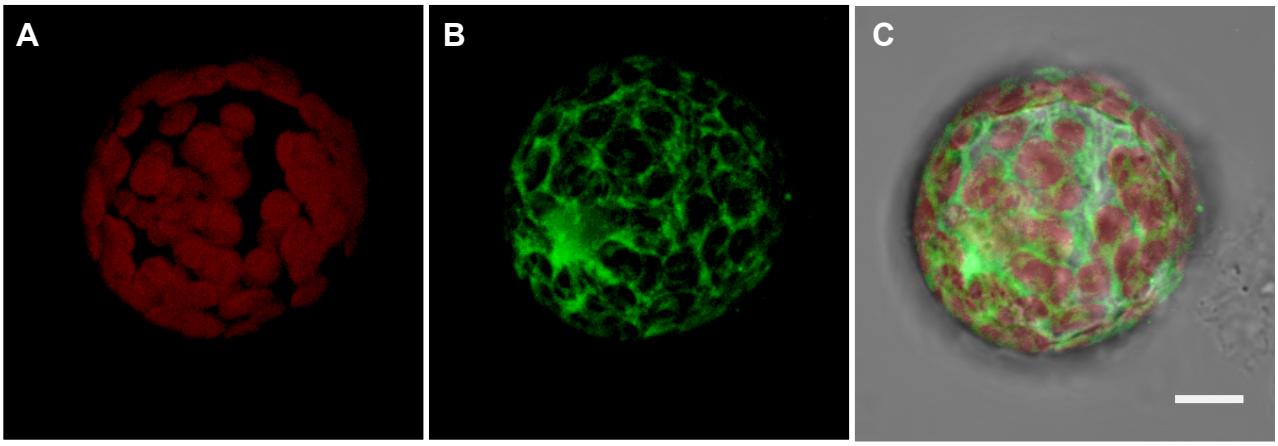



**Figure 29. Subcellular localization of PR5 (35S:GFP-PR5) in *N. benthamiana* protoplast.** (**A**) Red color represents chlorophyll autofluorescence, (**B**) green color represents GFP fluorescence and (**C**) shows merged transmitted light image of protoplast. Scale bar: 10 µm.

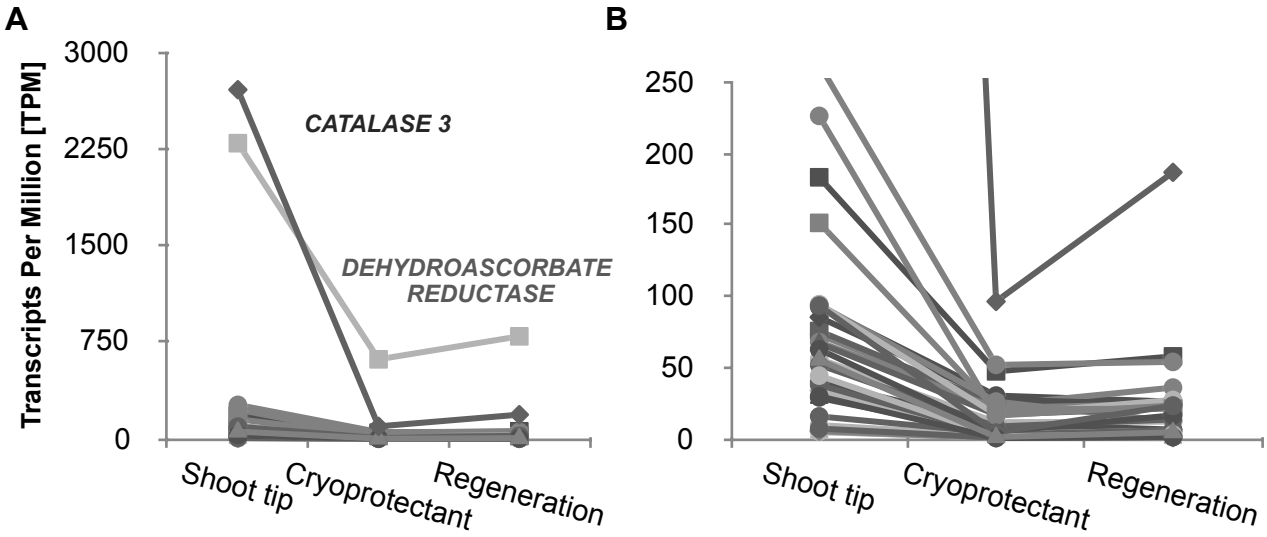



**Figure 30. Changes in transcript abundance of genes related to redox regulatory pathways during cryopreservation.** (**A**) The analysis identified 33 transcripts on the basis of a $\log_2$ fold change threshold ≥ 1.5 in phase II over phase I in WT shoot tips. (**B**) Zoom in the range from 0 to 250 TPM (Transcript per million reads). Phase I: shoot tip preparation; Phase II: cryoprotectant treatment (PVS2); Phase III: regeneration (1st day). *CATALASE 3*, *DEHYDROASCORBATE REDUCTASE*.

*REDUCTASE* (*At1g19570*) (**Figure 30A,B**). A detailed list of all depicted redox-dependent transcripts is listed in **Appendix Table 3**.

A $H_2O_2$ *in vivo* monitoring system was established to investigate the relationship between oxidative burst and cytosolic *PR5* expression after shoot tip preparation and the early phase of dehydration.

### 3.2.2.3. $H_2O_2$ *in vivo* monitoring confirmed occurrence of ROS after shoot tip preparation

The role of oxidative stress during the different steps of cryopreservation was investigated monitoring relative differences in cytosolic $H_2O_2$ concentration in WT plants harboring the pH2GW7:c-roGFP2-Orp1 construct (Col-roGPF2-Orp1).

The use of the reduction–oxidation-sensitive GFP2 (roGFP2) enable real-time, non-disruptive, compartment-specific measurement, unaffected by pH changes (Morgan *et al.*, 2011; Schwarzländer *et al.*, 2008). Fused to an Orp1 protein, a highly sensitive thiol peroxidase that is oxidized by $H_2O_2$ (Delaunay *et al.*, 2002; Meyer, 2008), relative differences in $H_2O_2$ concentration among the range of induced cryostressors were observed. This *in vivo* sensor offers advantages since traditional $H_2O_2$ measurements suffered from the problem of specificity and spatiotemporal resolution. The use of

redox-sensitive dyes like 2′,7′-dihydrodichlorofluoroscein lacks in specificity and the dye react irreversibly with cytochrome c, metals, and peroxidases (Tarpey *et al.*, 2004).

The principle of roGFP2 measurements is depicted in **Figure 31A**. When measuring fluorescence emission at 510 nm, roGFP2 exhibits excitation maxima at both 405 nm and 488 nm. The amplitude of both excitation maxima depends on the oxidized state of roGFP2. While oxidation increases the intensity of the 405 nm peak, the intensity of the 488 nm peak is decreased (Morgan *et al.*, 2011).

*In situ* performance of roGFP2-Orp1 was evaluated, adding either $H_2O_2$ or DTT to *Arabidopsis* seedlings, to drive the roGFP2-construct towards either fully oxidized or reduced forms (**Figure 31B**).

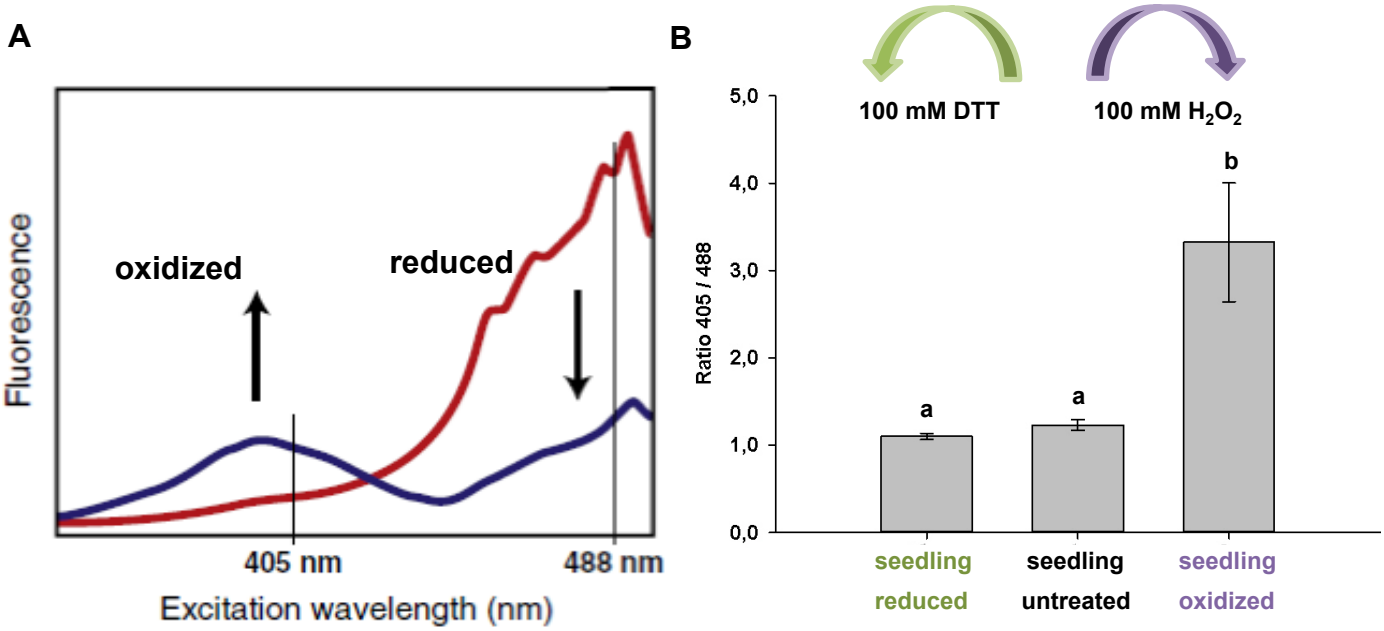



**Figure 31. Principle of $H_2O_2$ *in vivo* measurement.** (**A**) Changes in excitation maxima based on redox state (from *Morgan et al., 2011*) (**B**) Performance of Col-roGFP2-Orp1 seedlings, adding either 100 mM $H_2O_2$ or 100 mM DTT to *Arabidopsis* seedlings after 10 min incubation. The error bars indicate the SD ($n$=10). Statistical significance was analyzed with one-way ANOVA followed by Holm-Sidak post-hoc. Mean values marked by the same letter did not differ significantly from one another ($P \leq 0.05$).

As oxidative stress is often accompanied by membrane injury, the step after the shoot tip preparation was further analyzed with regard to changes in the oxidative burst (**Figure 32**). Relative $H_2O_2$ concentrations were higher in parts of the seedlings where leaves or roots were excised (seedling cut). At the same time, uncut parts of the seedling (seedling uncut) showed hydrogen peroxide concentrations similar to control seedling. Relative $H_2O_2$ level at the cutting sites dropped down, 30 min after wounding (seedling cut 30 min) but showed still higher levels of relative $H_2O_2$ concentration compared to control seedling (**Figure 32A,B**). These results revealed a precise

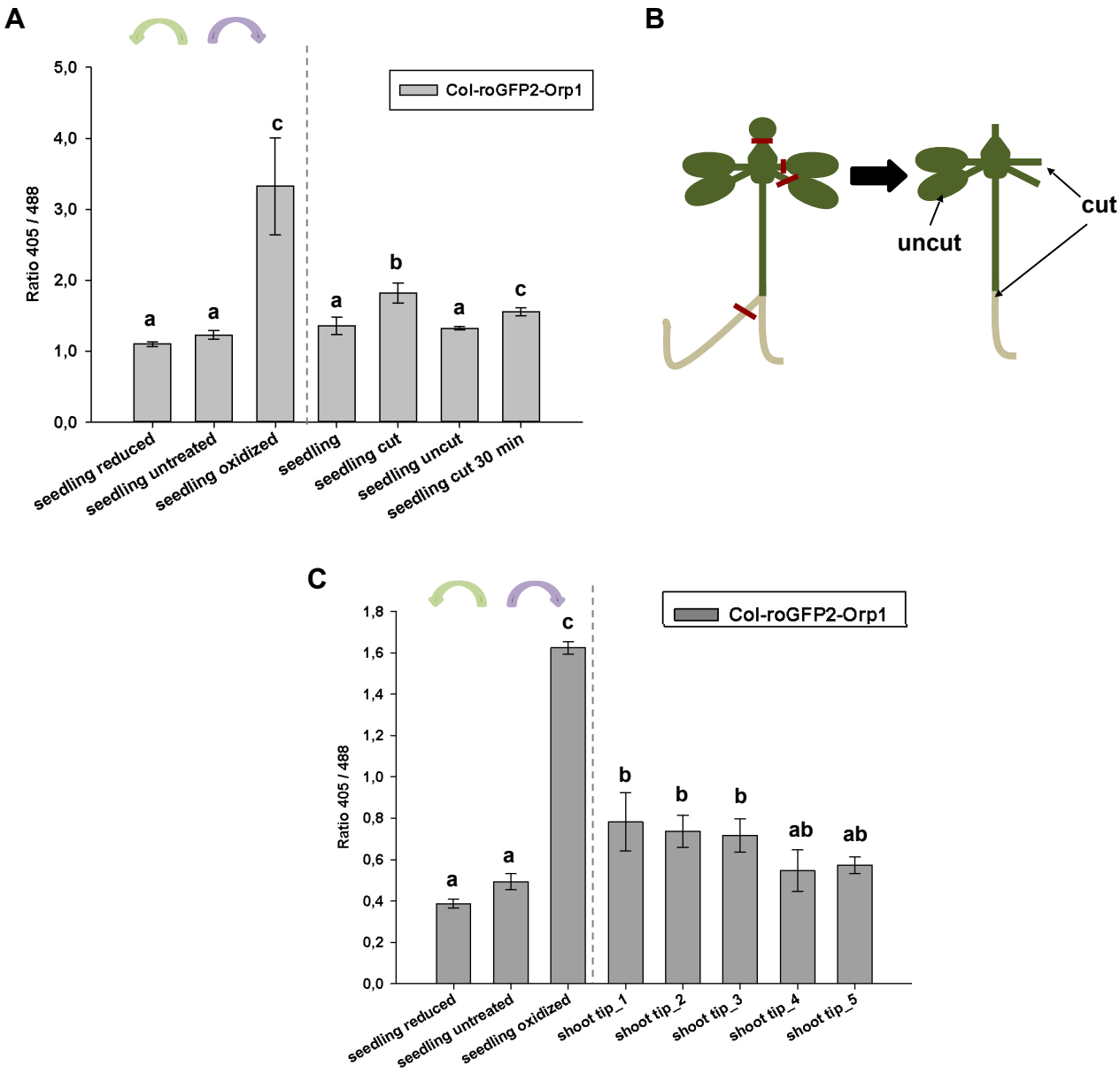



**Figure 32. Redox state of pH2GW7:c-roGFP2-Orp1 in the cytosol of *Arabidopsis* explants after shoot tip preparation.** (**A**) Relative differences in cytosolic $H_2O_2$ levels in Col-roGFP2-Orp1 seedlings indicated a precise distinction between cut and uncut parts of seedlings. (**B**) Schematic illustration of cutting sites of *Arabidopsis* seedlings. (**C**) Relative differences in cytosolic $H_2O_2$ levels among shoot tips after preparation in Col-roGFP2-Orp1 seedlings. Performance of Col-roGFP2-Orp1 seedlings was evaluated, adding either 100 mM $H_2O_2$ or 100 mM DTT. The error bars indicate the SD ($n$=10). Statistical significance was analyzed with one-way ANOVA followed by Holm-Sidak post-hoc. Mean values marked by the same letter did not differ significantly from one another ($P$≤0.05).

distinction between cut and uncut tissue of seedlings dependent on the level of produced hydrogen peroxide after tissue rupture.

Further experiments showed that $H_2O_2$ formation was always boosted in shoot tips, directly after the preparation step compared to untreated seedlings (**Figure 32C**). However, the strength of relative $H_2O_2$ concentration differed significant among prepared shoot tips and might be dependent on the level of tissue wounding.

### 3.2.2.4. Cytosolic $H_2O_2$ level might correlate with the transcript expression of *PR5*

WT and *pr5* loss of function explants, harboring the pH2GW7:c-roGFP2-Orp1 construct (WT-/*pr5*-roGPF2-Orp1) were used to monitor relative changes among shoot tip preparation, dehydration and PVS2 treatment (**Figure 33**). Compared to control seedlings (seedling untreated), each step of cryopreservation induced enhanced levels of hydrogen peroxide in WT and in *pr5* knockout shoot tips.

The basic level of hydrogen peroxide in *pr5*-roGPF2-Orp1 shoot tips was significant elevated compared to Col-roGPF2-Orp1. Shoot tip preparation, dehydration, as well as PVS2 treatment triggered the production of $H_2O_2$ to similar conditions when seedlings were fully oxidized.

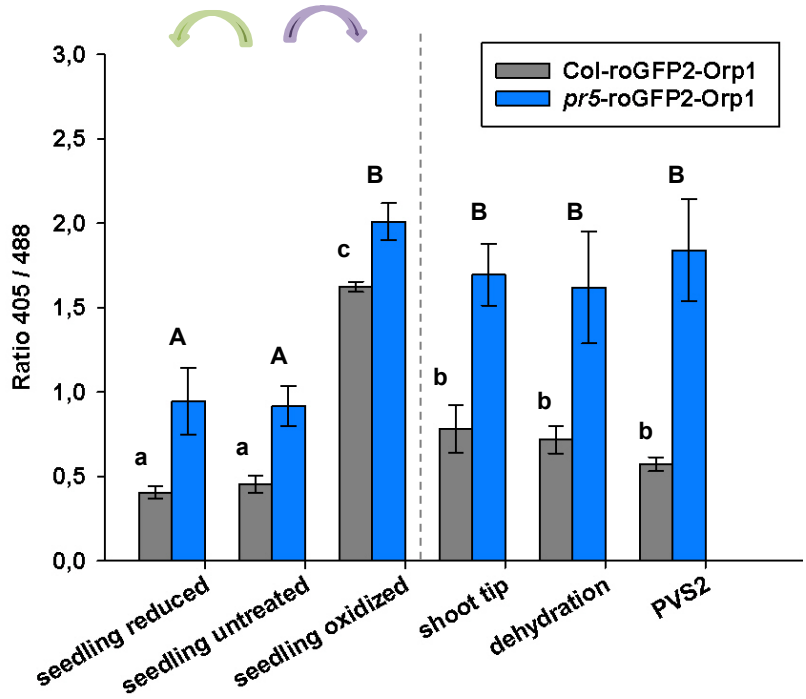



**Figure 33. Redox state of pH2GW7:c-roGFP2-Orp1 in the cytosol of *Arabidopsis* WT and *pr5* KO plants after shoot tip preparation, dehydration and cryoprotection (PVS2).** Performance of Col-roGFP2-Orp1 and *pr5*-roGFP2-Orp1 seedlings was evaluated, adding either 100 mM $H_2O_2$ or 100 mM DTT. The error bars indicate the SD ($n$=10). Statistical significance was analyzed with one-way ANOVA followed by Holm-Sidak post-hoc. Mean values marked by the same letter did not differ significantly from one another ($P \leq 0.05$). Small letter: Col-roGFP2-Orp1; Capital letter: *pr5*-roGFP2-Orp1.

### 3.2.2.5. The transcription factor *WRKY22* acts as a putative upstream regulator of *PR5* during the early phase of dehydration

Since the *PR5* promotor sequence contains W box (C/T) TGAC (T/C) motifs to interact with WRKY transcription factors (Kaur *et al.*, 2017), a putative function of *PR5* as a downstream target of *WRKY22* was suggested. *In silico* analysis using PlantPAN2.0

software showed four W-box motif elements in the predicted promotor sequence, 1000 bp upstream from the *PR5* gDNA sequence (**Figure 34A**).

If *WRKY22* acts as an upstream regulator of *PR5*, differences in the transcript expression during cryopreservation were suspected. qRT-PCR confirmed that the *PR5* transcript expression depends on the presence of a functional *WRKY22* transcript (**Figure 34B,C**). In WT explants, *WRKY22* transcription increased significantly after oPVS2 treatment and decreased during regeneration (**Figure 12, 34B**). Relative abundance of *WRKY22* transcript in *pr5* mutants showed the same expression pattern as in WT, but *WRKY22* transcript was significant induced after the dehydration step (**Figure 34B**).

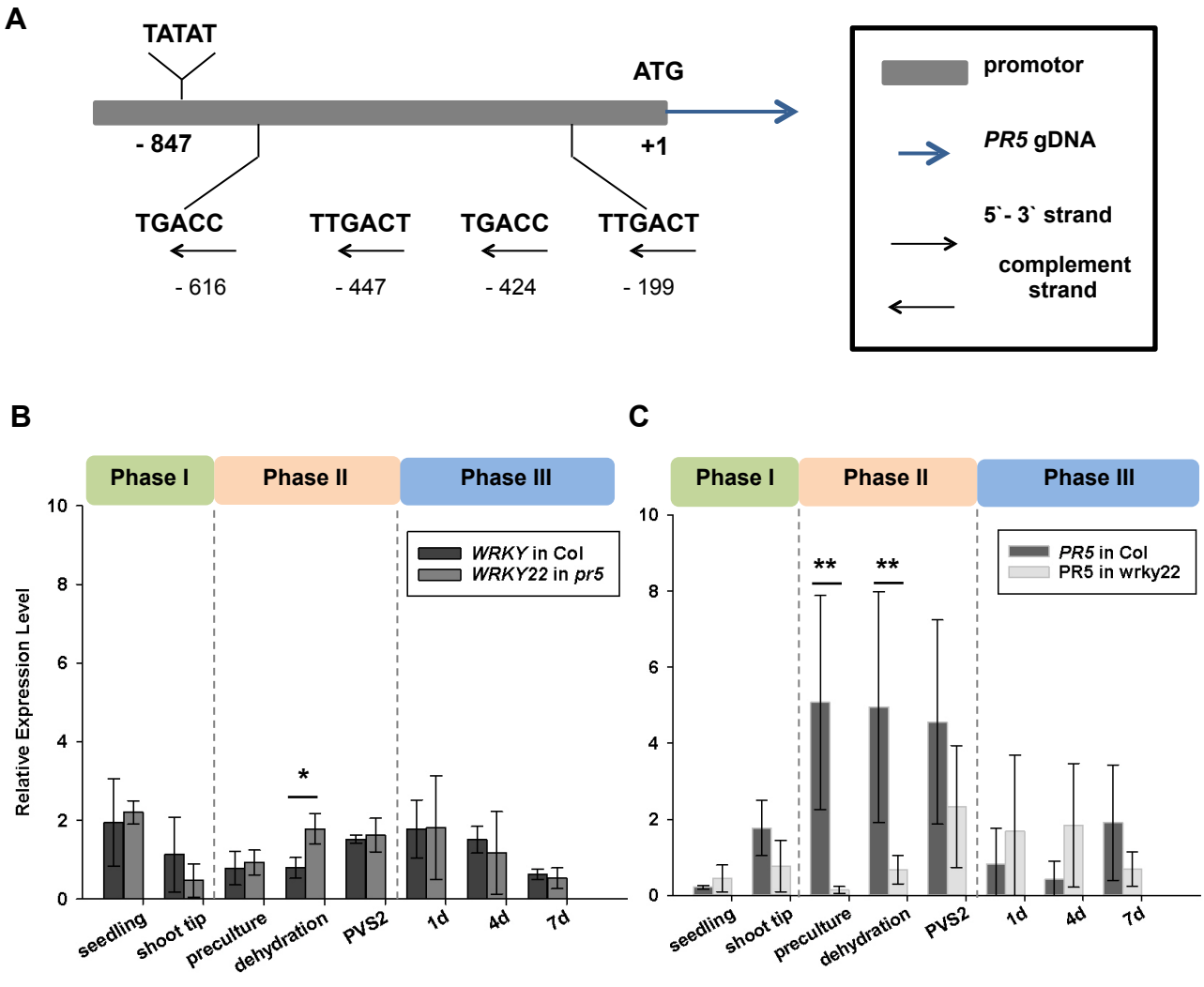



**Figure 34. *PR5* transcript expression dependent on the transcription factor *WRKY22*.** (**A**) Scheme of the predicted promotor region 1000 bp upstream of the *PR5* gDNA sequence. The position of the putative W-box (C/T) TGAC (T/C) elements is indicated. Relative abundance of (**B**) *WRKY22* transcript present in either Col-0 or *pr5* mutant explants or **(C)** *PR5* transcript present in either Col-0 or *wrky22.1* mutant explants sampled at certain stages of the cryopreservation/recovery process. The error bars indicate the SD (*n*=3). Statistical differences between transcript expression in Col-0 and mutant were calculated using student t-test (* $P \leq 0.05$; ** $P \leq 0.01$).

*PR5* transcript was significantly induced in phase II and decreased during phase III in WT (**Figure 28; Figure 34C**), indicating that *PR5* is likely involved in the response to wound-induced stress and the early phase of dehydration. However, in the absence of a functional copy of *WRKY22*, as it was shown for the *wrky22* mutants (**Figure 11A,B**), the *PR5* transcript was significantly reduced during phase II comprising all dehydration steps compared to WT expression (**Figure 34C**). *PR5* transcript abundance in *wrky22* KO mutant shoot tips showed no significant differences compared to WT transcript levels during recovery (**Figure 34C**).

Finally the effect of knocking out both genes, *PR5* and *WRKY22,* on the performance of cryopreserved explants was explored by exposing T-DNA double knockout lines (*wrky22*x*pr5*) to the PVS2 a cryostorage protocol (**Figure 35**). The vast majority (98 %) of WT shoot tips regenerated into viable plantlets after cryogenic treatment. As shown in **Figure 9, 11, 27** and **35** the lack of a fully functional copy of either *WRKY22* or *PR5* resulted in a significantly impaired level of regeneration. Also both lines of double knockout explants (*wrky22*x*pr5* and *pr5*x*wrky22*) showed significant impaired recovery compared to WT and moreover, the viability was on the same level (70 %) as it was observed for single knockout *pr5* (60 %) and *wrky22* (65 %) (**Figure 35A**).

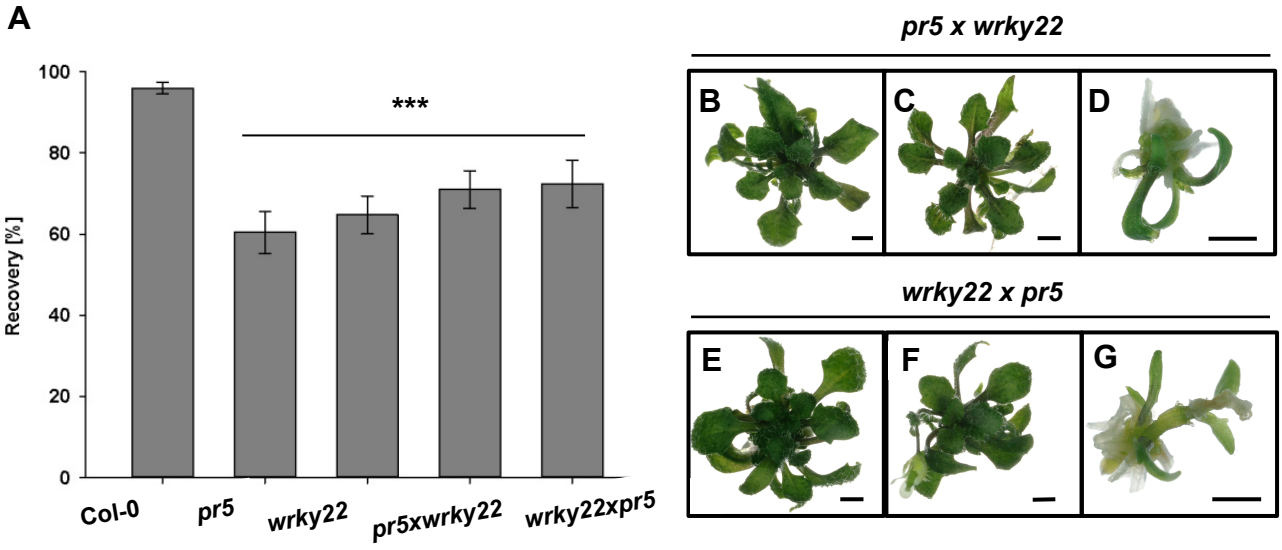



**Figure 35. Regeneration of *Arabidopsis* single mutant and double mutant plantlets after cryopreservation.** (**A**) Percentage of recovered plantlets of Col-0 (WT), *pr5*: *PATHOGENESIS RELATED GENE 5*, *wrky22*: *WRKY22* transcription factor, and double knockout mutants: *pr5*x*wrky22*; *wrky22*x*pr5*. The performance of the mutants was compared to that of WT using the Win Fisher test (***: $P \leq 0.001$; $n \geq 90$). (**B-G**) The appearance of recovered plantlets derived from shoot tips of (**B-D**) transgenic *pr5*x*wrky22*, (**E-G**) transgenic *wrky22*x*pr5* double mutant. Scale bar: 1 mm.

Phenotypic analysis showed that recovered plantlets from *wrky22*x*pr5* and *pr5*x*wrky22* exhibited a distinct phenotype: 60-65 % of the plantlets retained a WT phenotype (**Figure 35B,C,E,F**), while 40 % developed incomplete leaves and roots, produced some callus material or stayed green without any further development (**Figure 35D,G**).

# CHAPTER 4. DISCUSSION

## 4.1. New insights in molecular mechanisms underlying cryopreservation

Achieving a high level of post-cryogenic viability is important to ensure *ex situ* preservation of currently endangered plant species and maintain biodiversity. This study showed that:

- *Arabidopsis* shoot tips can overcome cryo-induced stress response accompanied with high post-cryogenic recovery.
- The established PVS2 Droplet Vitrification protocol resulted in high viability after cryopreservation among selected *Arabidopsis* wildtype accessions.

Obtaining a high viability requires explants to respond to a complex trait of different abiotic stressors like wounding and the exposure to osmotic, chemical and low temperature during critical steps of cryopreservation. To my knowledge, this is the first study were shoot tips of *Arabidopsis* were used to address the fundamental problems of the multiple stress response during cryopreservation on the molecular level. As depicted in **Figure 36**, a transcriptomic approach and further molecular characterization of WT and T-DNA insertion plants unraveled molecular mechanisms underlying cryopreservation after shoot tip preparation (phase I), cryoprotectant treatment (phase II) and first day of recovery (phase III) in WT:

- Shoot tip preparation (**phase I**) induced defense-related stress responses mediated by ROS signaling ($H_2O_2$) and phytohormone-mediated processes (SA and JA) on the transcriptomic level, dependent on the transcript abundance of *WRKY22* and *PR5* (**Figure 36A**).

- Cryoprotectant treatment (**phase II**) initiated osmotic changes enabling a reversible degradation on the structural level of meristematic cells. This was associated with an increased transcript expression of genes involved in RNA processing in WT shoot tips.
- Cryostressors induced phytohormone-mediated defense (SA and JA) and drought (ABA) stress response in WT shoot tips (**Figure 36B**).

- PVS2-induced drought-stress response is regulated by changes in stomatal aperture.
- Developmental processes during regeneration (**phase III**) were mainly driven by auxin (IAA)-signaling, whereas defense response was repressed. This indicates a growth-defense tradeoff in the early regeneration process (**Figure 36C**).

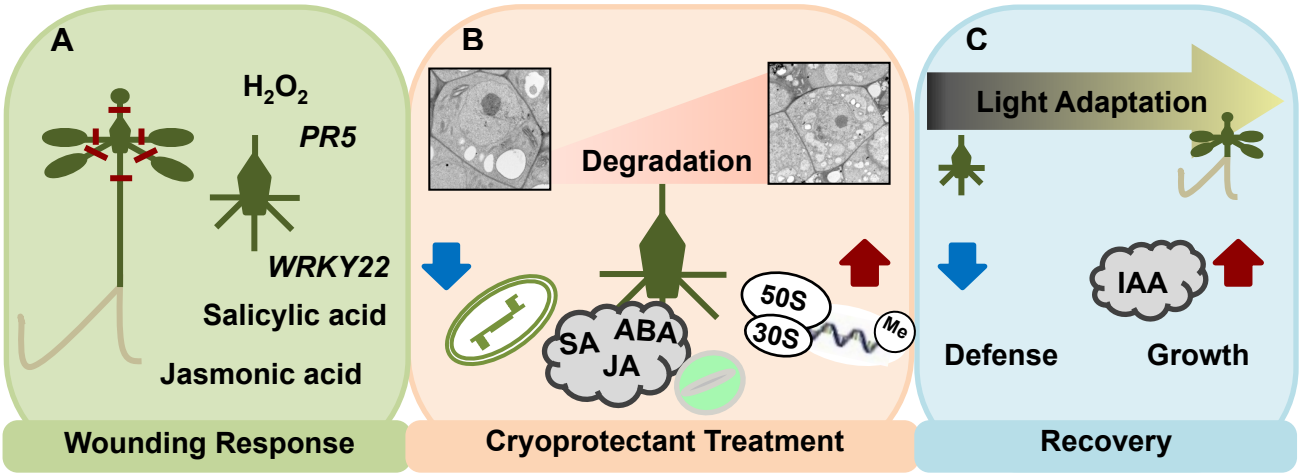



**Figure 36. Schematic overview of the molecular mechanisms underlying cryopreservation. (A)** Wounding response as a consequence of shoot tip preparation in the initial phase. **(B)** Cryoprotectant-induced changes in ultrastructure, hormone signaling, RNA processing/methylation and primary metabolism in terms of photosynthesis. **(C)** During the first day of regeneration a growth-defense tradeoff was observed, as well as an over-representation of auxin (IAA) induced pathways. ABA: abscisic acid; Me: Methylation; JA: jasmonic acid; *PR5*: *Pathogenesis Related Gene 5*; SA: salicylic acid.

Moreover, this study provided great insight into basic function of *WRKY22* and *PR5* during processes where multiple abiotic stressors accompanied simultaneously.

- In general, the response of the *wrky22* mutant comprised a less organized stress response.
- The loss of the functional *WRKY22* transcript limited the response to ROS production, phytohormone-mediated defense (SA and JA) and drought stress (ABA) response in mutant shoot tips in phase I and phase II.
- *WRKY22* is involved in the regulation of stomata closure an important mechanism of drought and defense adaptation.

- *PR5* transcript expression is dependent on the level of reactive oxygen species ($H_2O_2$) after tissue rupture.

- *PR5* promotor region presents W box motifs to interact with *WRKY22* TF.
- *PR5* transcript expression depends on the presence of the functional *WRKY22* transcript.
- *WRKY22* was identified as a putative upstream regulator of *PR5* during osmotic dehydration.

### 4.2. Droplet vitrification methods in *Arabidopsis* cryobiology

The tolerance of shoot tips to an extended exposure to PVS2 suggested that vitrification solutions, which comprise DMSO as cryoprotectant, had no severe damaging effect on recovery, as it was suspected in previous studies (Panis and Lambardi, 2005; Volk *et al.*, 2014). Phases of PVS2 incubation up to 1 h (PVS2 a) even improved recovery compared to 20 min PVS2 exposure (PVS2 b). Furthermore, non-cryopreserved (LN-) samples always displayed more than 95 % recovery, which indicated that the viability after cryopreservation was independent and not affected by the toxicity of the highly concentrated cryoprotectant solutions employed. PVS3-treated shoot tips showed lower recovery compared to PVS2 treatments. Increasing the PVS3 exposure duration, higher recovery might be achieved as suggested in recent literature (Sakai *et al.*, 1990; Yi *et al.*, 2012).

Acclimated explants exhibit enhanced recovery compared to non acclimated, indicating that accumulated compounds during hardening phase have benefit effects for recovery. Shoot tips treated with the DMSO Droplet-Freezing protocol had the lowest recovery and Droplet-Freezing was the only method, which did not induce a glass transition during cooling. These results indicated that vitrification in combination with an acclimation during the growing phase is crucial for high post-cryogenic recovery of cryopreserved *Arabidopsis* shoot tips.

High recovery is, among other factors, largely dependent on the cryostored plant species, tissue type, and genotype. Therefore, a stable and genotype-independent *Arabidopsis* cryopreservation protocol is important to get deeper insights into the molecular background of cryopreservation. Previous studies revealed that recovery varies from 58 % to 98 % for *Arabidopsis* shoot tips and seedlings (Ren *et al.*, 2015; Ren *et al.*, 2013; Wang and He, 2009). The most promising method (PVS2 a) was applied to a range of WT accessions and yielded 89 % to 99 % recovery among the different tested genotypes. Consequently, the *Arabidopsis* model appears to represent a suitable platform for identifying the mechanistic basis of the response to the stresses imposed by the cryopreservation process.

### 4.3. The WT explant's transcriptome response to cryostressors

It has been proposed that recovery post-cryopreservation is largely compromised by a build-up of reactive oxygen species, together with a reduced capacity to produce the detoxifying antioxidant enzymes (Chen *et al.*, 2015; Gross *et al.*, 2016; Ren *et al.*, 2015; Uchendu *et al.*, 2010). The abundance of transcript from the redox stress marker genes *ATH8* (*At1g69880*), *AHB1* (*At2g16060*), *APX2* (*At3g09640*) and *GPX7* (*At4g31870*) was promoted by the cryoprotectant treatment, but most of the set of known reactive oxygen species marker genes were down-regulated in the WT explants, and only marginally altered in the *wrky22* mutant ones. This suggests that the influence of reactive oxygen species and the harmful effect on lipid peroxidation is at best a minor one. It is more likely that ROS, like hydrogen peroxide, act as early signal molecules to induce for instance defense response after tissue rupture.

The PVS2 reagent combines a number of different cryoprotectant substances, some of which are potentially toxic for meristematic cells (Volk *et al.*, 2006). Although the post-cryogenic recovery is not affected by the exposure to toxic cryoprotectants, DMSO inhibits electron transport in the chloroplast (Reeves and Hall, 1977). Therefore, it can be expected to affect the transcription of genes associated with photosynthesis. To reduce its chemotoxicity, the cryoprotectant treatment is typically conducted in the dark at a low temperature, conditions which suppress photosynthesis. Osmotic stress, resulting from the partial dehydration of the explant required to avoid the formation of ice crystals during the cooling step, also tends to repress the transcription of genes involved in photosynthesis. It is known that salinity treatment, which imposes osmotic stress, can induce stomatal closure, the inhibition of $CO_2$ fixation and a reduced flux of electrons through photosystem II (PSII) (Kilian *et al.*, 2007; Stepien and Johnson, 2009); here, the intensity of transcription in the treated WT explants was reduced with respect to PSII-associated genes like *PSBR* (*At1g79040*) and *LHCB2.3* (*At3g27690*) (**Table 9**). Thus, the suggestion is that chemotoxicity and osmotic stress represent significant components of the overall stress imposed by the cryostressors.

Although there is some evidence that the cryoprotectant treatment can change DNA methylation patterns (Hao *et al.*, 2001; Harding, 1994, 2004; Johnston *et al.*, 2009), the present data indicated that the cryostress response is mainly involved in RNA modification. RNA processing is known to be a component of both the adaptation to low temperature and drought, as well as of development (Baldridge and Contreras, 2014; Hébrard *et al.*, 2013; Zhong *et al.*, 2008). It is possible that for some genes, non-functional transcripts are produced under normally encountered environmental

conditions, but that active variants are generated when the tissue is challenged by stress (Egawa *et al.*, 2006; Matsukura *et al.*, 2010; Oh *et al.*, 2011; Qin *et al.*, 2007; Sakamoto *et al.*, 2013; Wang *et al.*, 2013). Thus alternative splicing could be envisaged to represent a strategy for the explant to adapt to the stresses imposed by cryostorage, allowing it to initiate the degradation of meristematic cells.

### 4.4. The *WRKY22* transcription factor – a regulator of multiple stress responses in *Arabidopsis* shoot tips during cryopreservation

Consistent with what has been reported in the literature (Kloth *et al.*, 2016), *WRKY22* was shown here to be involved both in the synthesis of the cell wall (**Appendix: Data Set 1**) and the SA-mediated stress response (**Figure 21A**). Most of the changes induced in the transcriptome took place during phase II, possibly reflecting a delayed wounding response. The altered nature of the SA-mediated defense response may have arisen through cross-talk with *WRKY53* and *WRKY70*, both of which are known to act as regulators of SA-mediated gene transcription (Kloth *et al.*, 2016; Li *et al.*, 2004; Miao *et al.*, 2007; Wang *et al.*, 2006a).

*WRKY22* participated in the explants' adaptation to osmotic stress by promoting stomatal closure (**Figure 24**). Since both *WRKY53* and *WRKY70* have been identified as repressors of stomatal opening (Li et *al.,* 2013a; Sun and Yu, 2015), the implication is that *WRKY22* co-operates with these two transcription factors in the context of the explants' adaptation to osmotic stress. The suggestion is that the open stomata phenotype, shown by the *wrky22* mutant shoot tips, would allow for an increased fixation of $CO_2$, driving electron transport through PSII under certain conditions. However, open stomata results in parallel in a greater volume of $H_2O$ loss and a drought stress response sensitive phenotype in *WRKY22* loss of function plants beyond the process of cryopreservation (**Figure 25C**).

The schematic model presented in **Figure 37** summarizes key aspects of the *WRKY22*–mediated regulation of both the osmotic stress and defense responses. The PVS2 treatment and excision of the explant trigger stomatal closure, mediated by the activity of *WRKY53* and *WRKY70*, while at the same time, the wounding response is orchestrated by genes responding to a SA signal. The *wrky22* mutant's open stomata phenotype enhances the volume of $CO_2$ fixation, driving changes in the transcription of genes encoding PSII. In parallel open stomata promotes the loss of $H_2O$, resulting in the drought stress phenotype of *wrky22* mutant plants.

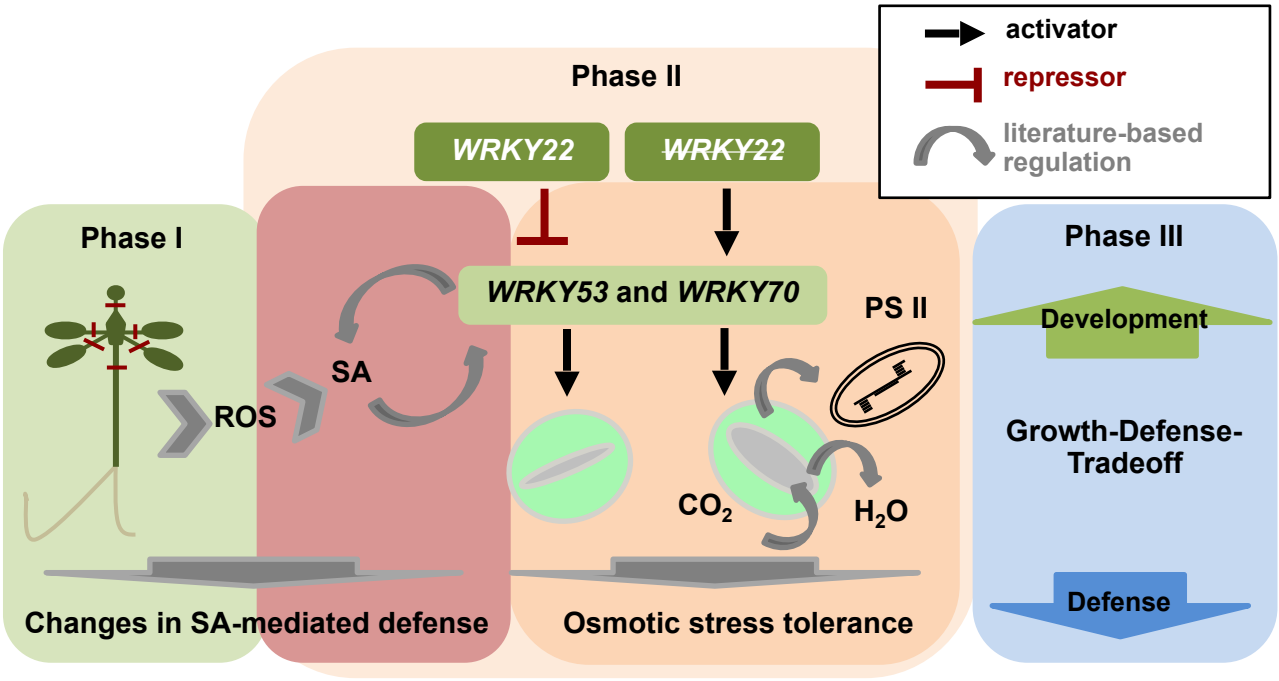



**Figure 37. Proposed function for *WRKY22* during the cryopreservation process.** *WRKY22* suppresses the transcription of *WRKY53* and *WRKY70*, resulting in an altered salicylic acid-mediated wounding response; the osmotic stress response is changed as a result of altered stomatal opening behavior. The open stomata phenotype exhibited by the *wrky22.1* mutant results in a greater volume of $H_2O$ loss and $CO_2$ fixation and a change to the chloroplasts' capacity. A higher energy demand may limit the trade-off between growth and defense, resulting in the mutant explants suffering a compromised level of post-cryopreservation recovery.

During regeneration, the defense response is suppressed in order to free up resources for the purpose of development. Thus, the amount of energy provided by the chloroplasts represents a limiting factor for explant regeneration and thereby affects the rate of recovery. During recovery, a number of genes involved in auxin-driven growth or in histone modification showed higher abundance in the *wrky22* mutant explants, while certain defense response genes were lower expressed (**Figs 18, 21B**, **Appendix: Data Set 1**): this represents a strategy whereby a choice is made between growth and defense (Huot *et al.*, 2014). A misbalanced stress response in *wrky22* mutants in phase II results in a significant proportion of the rewarmed explants being able to form only non-differentiated callus rather than new shoot material.

### 4.4.1. *WRKY22* links drought and defense response via stomatal closure

In **Figure 23B** and **37** *WRKY22* regulates multiple responses simultaneously like phytohormone-mediated osmotic stress adaptation and wounding response via stomata closure.

## DISCUSSION

Guard cell closure during abiotic stress ensures gas exchange of $CO_2$ and water vapor, required for photosynthesis and water homeostasis. Therefore the size of stomata is regulated by the osmotic pressure of the two surrounding guard cells via ABA as a predominant regulator in response to changes in environmental conditions like drought stress (Montillet and Hirt, 2013).

Moreover, stomata closure can also be linked to plant innate immune system (Melotto *et al.*, 2006). For instance, *WRKY22* is a component of the MAPK mediated plant defense response against pathogens like *P. syringae* but also fungal pathogens (Asai *et al.*, 2002; Göhre *et al.*, 2012). *P. syringae* is attracted towards open stomata and upon the detection of the bacterium, the plant triggers stomata closure by SA and ABA signaling pathways. Coronatine, a compound produced by *P. syringae* promotes stomata reopening after certain incubation period. Motile bacteria invade the apoplastic space of mesophyll cells and effectors suppress the expression of defense genes mediated by immune response like WRKY transcription factors (Melotto *et al.*, 2006; Schulze-Lefert and Robatzek, 2006).

A regulatory function in biotic and abiotic stresses is not restricted to *WRKY22*. In *Arabidopsis*, *WRKY25* and *WRKY33*, are considered as positive regulators in response to salt stress (Jiang and Deyholos, 2009). In addition, *WRKY33* is a positive regulator of resistance to the necrotrophic fungal pathogens (Zheng *et al.*, 2006) and towards *Botrytis cinerea* infection (Birkenbihl *et al.*, 2012). Also *WRKY25* was described to be involved in defense against *P. syringae* (Zheng et al., 2007). Within the WRKY transcription factor family, *WRKY25* from *Gossypium hirsutum* (*GhWRKY25*), as well as *GhWRKY27a* are involved in both response to drought stress and resistance (Liu *et al.*, 2016; Yan *et al.*, 2015). Even beyond the WRKY TF family, *PR*-genes in potato induced osmotolerance, as well as biotic stress response with benefit effects on post-cryogenic viability (El-Banna *et al.*, 2010).

Due to the induced *PR5* transcript expression after shoot tip preparation and during dehydration (**Figure 28**), a putative role in wounding response, as well as stress response towards osmotic adaptation was suggested. Since *PR5* was proven to act as a putative downstream target of *WRKY22* (**Figure 34**), a specific role in the stomata-dependent defense response was expected. This hypothesis was affirmed by a PR5-specific expression in the cytosol of stomatal guard cell, confirmed via PR5:promotor-GUS construct during drought stress response (Liu *et al.*, 2013).

Based on the results of this study and known literature a schematic model presented in **Figure 38** summarizes key aspects of the *WRKY22*-dependent *PR5*

transcript expression with regard to defense and osmotic stress response in stomata. As described in the previous section, open stomata upon osmotic changes in the environment, leads to increased $CO_2$ fixation and a loss of $O_2$ and $H_2O$. On the other hand, open stomata represents a lost barrier against bacterial invasion, which is accompanied with wounding response in nature.

*WRKY22* might counteract in two directions to avoid pathogen infection upon wounding. In order to induce early defense response, *WRKY22* represses open stomata via cross-communication with *WRKY53* and *WRKY70* (**Figure 37**). The expression of *PR5* in the cytosol of stomatal guard cells, as a late defense response, serves as a repressor of bacterial propagation or fungal invasion, since PR proteins are known to show antifungal function. Promoted production of reactive oxygen species ($H_2O_2$) during the defense response induces *PR5* transcript expression. The strength of *PR5* expression probably depends on the level of cell wounding and the accompanied

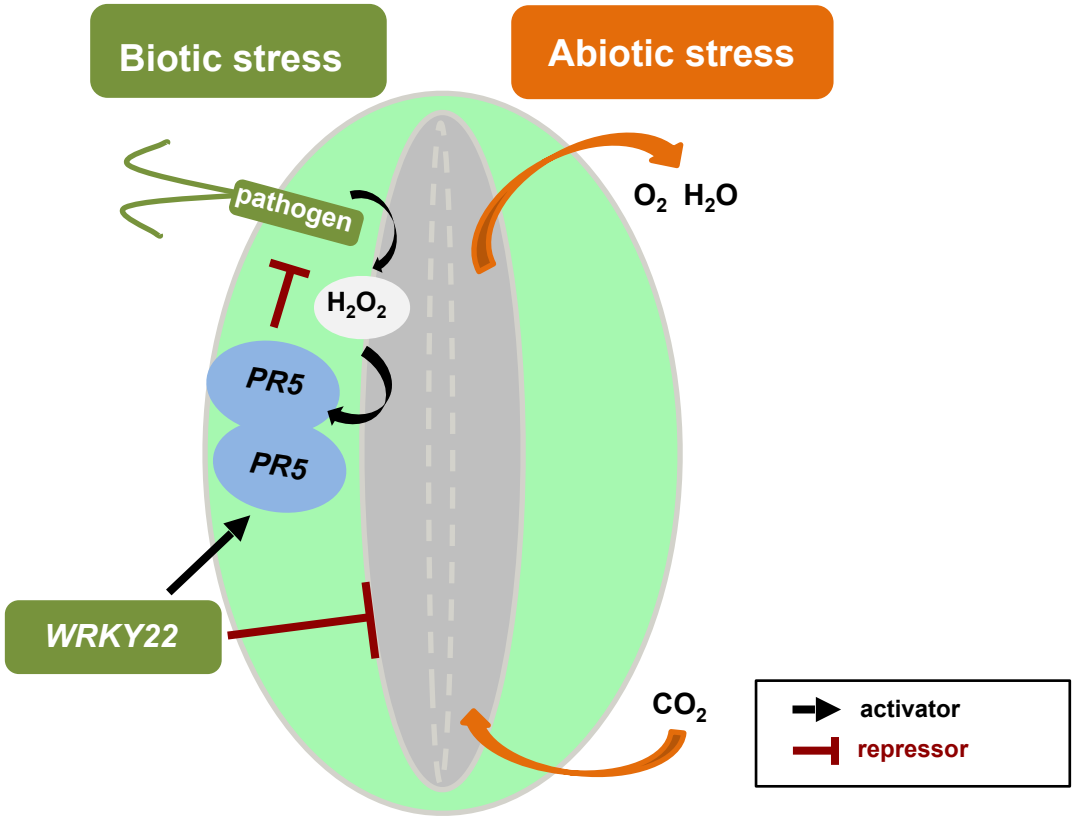



**Figure 38. The putative function of the *WRKY22*-dependent *PR5* expression in stomata.** Upon wounding or pathogen attack (biotic stress conditions), *WRKY22* represses open stomata and induces expression of *PR5* in stomatal guard cell of WT plants. *PR5* expression can be promoted by $H_2O_2$ after wounding and/or pathogen attack. Closed stomata have beneficial effects related to osmotic stress response (abiotic stress) with a reduced volume of $H_2O$ loss and $CO_2$ fixation.

$H_2O_2$ accumulation, since hydrogen peroxide levels were boosted in *pr5* loss of function mutant (**Figure 33**).

However, if *WRKY22* act as direct upstream regulator of *PR5* or interact in cross-communication with *WRKY53* and *WRKY70* needs to be verified in subsequent experiments. Since cryopreserved shoot tips face multiple abiotic stresses in parallel (phase I and phase II), the evolved network is a major advantage to overcome cryo-induced stressors with finally high post-cryogenic recovery.

### 4.4.2. The potential of so far unknown cryostressors

In this study, several stress adaptation mechanisms underlying cryopreservation under the control of *WRKY22* were identified. Some of them were already characterized in previous studies as indicated in **Figure 39**, but so far not in the context with cryostress.

*WRKY22* was described as a regulator of dark induced leaf senescence (Zhou *et al.*, 2011). It promotes susceptibility to aphids by modulating SA and JA signaling and

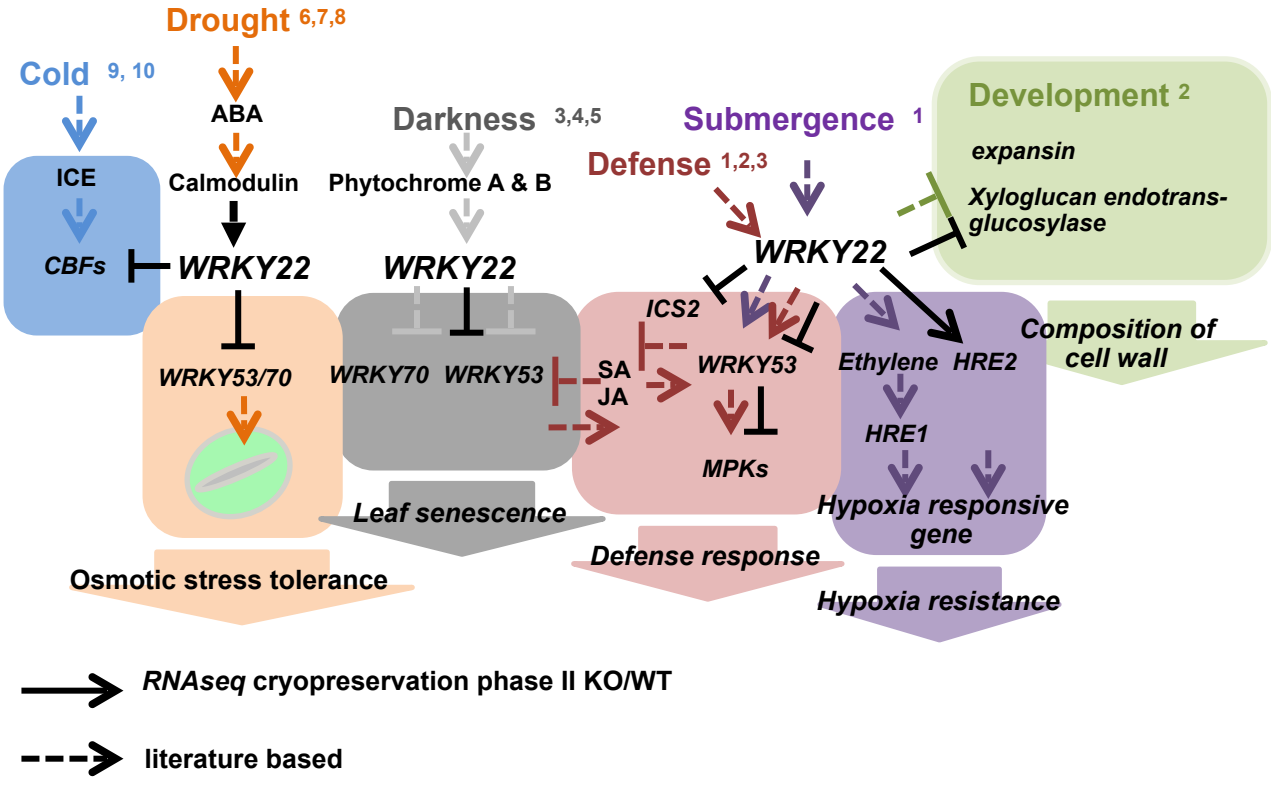



**Figure 39. Influence of *WRKY22* in many aspects of abiotic and biotic stress.** Simplified scheme of putative cryostress (cryoprotectant treatment) induced adaptation responses dependent on the transcription factor *WRKY22*.
[1] Hsu et al., 2013; [2] Kloth et al., 2016; [3] Miao and Zentgraf, 2007; [4] Zhou et al., 2011; [5] Zhang et al., 2015b; 6 Wu et al., 2017; [7] Li et al., 2013; [8] Sun and Yu, 2015; [9] Lee et al., 2005; [10] Park et al., 2015. *CBF*: *C-Repeat Binding Factor*; *HRE*: *Hypoxia Responsive Element*; *MPK*: *Mitogen-activated protein kinase*.

changes in the secondary cell wall composition in terms of expansins (Kloth *et al.*, 2016). Moreover the TF is involved in early cold stress response (Park *et al.*, 2015).

Beside the WRKY transcription factor family, interestingly, especially members of the AP2/ERF TF family appeared to act together with *WRKY22* during the cryopreservation process (**Figure 23**). This suggests that *WRKY22* interacts not only with members of the WRKY transcription factor family, but also cross-communicates with transcription factors from different families.

The products of the *CBF* genes are known to be involved in the adaptation to several abiotic stresses (Jaglo-Ottosen *et al.*, 1998; Liu *et al.*, 1998; Sakuma *et al.*, 2002), so may also contribute to the response to the cryoprotectant treatment. This notion is based on the fact that a higher rate of post-cryogenic recovery is correlated with a higher abundance of *CBF* transcript, as stated by Ren *et al.* (2015).

However, *CBF* transcription was only induced in the *wrky22.1* mutant. Thus *CBF*s are likely to be influential in supporting the viability of explants under sub-optimal condition and less organized stress response.

*WRKY22* provides submergence tolerance and activates ethylene signaling pathways via induction of *HRE2* transcript expression, a gene induced under hypoxic conditions (Hsu *et al.*, 2013). The role of hypoxia stress response during cryopreservation was previously described by Subbarayan *et al.* (2015). They could show that PVS3, a cryoprotectant solution, promoted oxygen deficiency in garlic shoot tip cryopreservation in correlation with changes in the primary metabolism. Thus, post-cryogenic viability was negatively affected in garlic accessions. As a consequence, low oxygen conditions, and the induced stress response-cascade should be considered as starting point to investigate putative cryostressors.

### 4.4.3. *WRKY22* forms transcription factor cluster to adapt to multiple stress responses

To regulate multiple stress responses simultaneously, an enormous interconnecting network evolved by plants. WRKY TFs work in cluster to mediate adaptation towards stress and development. A well investigated example for TF-cluster in *Arabidopsis* is the *WRKY18-40-60* cluster, induced in response to fungi (Rushton *et al.*, 2012). It participates in the SA and JA signaling-pathways (Brotman *et al.*, 2013; Chen *et al.*, 2010a). Moreover, this cluster regulates chloroplast-mediated retrograde signaling and acts as negative regulator of ABA signaling (Shang *et al.*, 2010).

As depicted in **Figures 37 and 39**, *WRKY22* regulated the transcript expression of *WRKY70* and *WRKY53* during cryoprotectant treatment (phase II). This raised the question whether *WRKY22* forms a *WRKY22-53-70* cluster to regulate crucial steps in biotic and abiotic stress response. Zhou *et al.* (2011) investigated that *WRKY22* positively regulates senescence and can influence the expression of its own gene and of *WRKY53* and *WRKY70*. Furthermore, aphid attack-mediated defense response is regulated by *WRKY53* in dependence of *WRKY22* (Kloth *et al.*, 2016). The transcriptomic dataset emphasized a putative *WRKY22-53-70* cluster for drought and defense response with regard to stress induced stomata closure.

### 4.5. Perception of cryostressors in the shoot apical meristem

Plants generate new tissue through the activity of undifferentiated stem cell, the meristem. Shoot tips contain the SAM, generates all aerial parts of the plants. Plants can adapt developmental processes to their environment, because their meristems adjust their activity (Aida and Tasaka, 2006). As depicted in **Figure 40**, auxin transport and biosynthesis can be regulated based on environmental conditions under the control of auxin transporters creating auxin maxima and minima within the SAM. Consequently, auxin is not homogeneously distributed, shows a dynamic transport and triggers differential gene expression and patterned growth (Vernoux *et al.*, 2010). Further, meristem function and developmental processes are promoted by the *Arabidopsis WUSCHEL* (*WUS*) gene. The transcription factor *WUS* is involved in a negative feedback loop with the *CLAVATA* (CLV) receptor kinase signaling cascade, whose activity limits the size of the pool of stem cells (Laux *et al.*, 1996).

Meristems have the possibility to generate plant structure, show autonomous function, and continue normal development when separated from surrounding plant tissue in optimized tissue culture. Early experiments demonstrated that SAM develop normally when detached from plants, if basic nutrient requirement is provided (Shabde and Murashige, 1977). Also cryopreservation of shoot apical meristem without surrounding tissue was successful, as it was shown for meristems of lily cultivars (*Lilium L.*) (Chen *et al.*, 2015) and *Musa* plants (Panis *et al.*, 1996). In meristem cryopreservation, shoot apical meristem act as a self-perpetuating source for regeneration of plantlets.

In this study the mechanisms underlying cryostress adaptation with regard to changes in the shoot tip transcriptome was investigated. Based on the dataset it was

not possible to distinguish between shoot apical meristem response and adaptation of primordia and surrounding shoot tip tissue.

The molecular characterization of the candidate genes *PR5* and *WRKY22* led to the conclusion that wounding dependent from the strength of cell injury and the accompanied $H_2O_2$ burst, as well as changes in osmolality reduced post-cryogenic recovery. These stressors are perceived in the meristem surrounding shoot tip tissue. But how do changes in the shoot tip stress response affect developmental processes in the shoot apical meristem could not be addressed with this study and need to be solved in subsequent experiments.

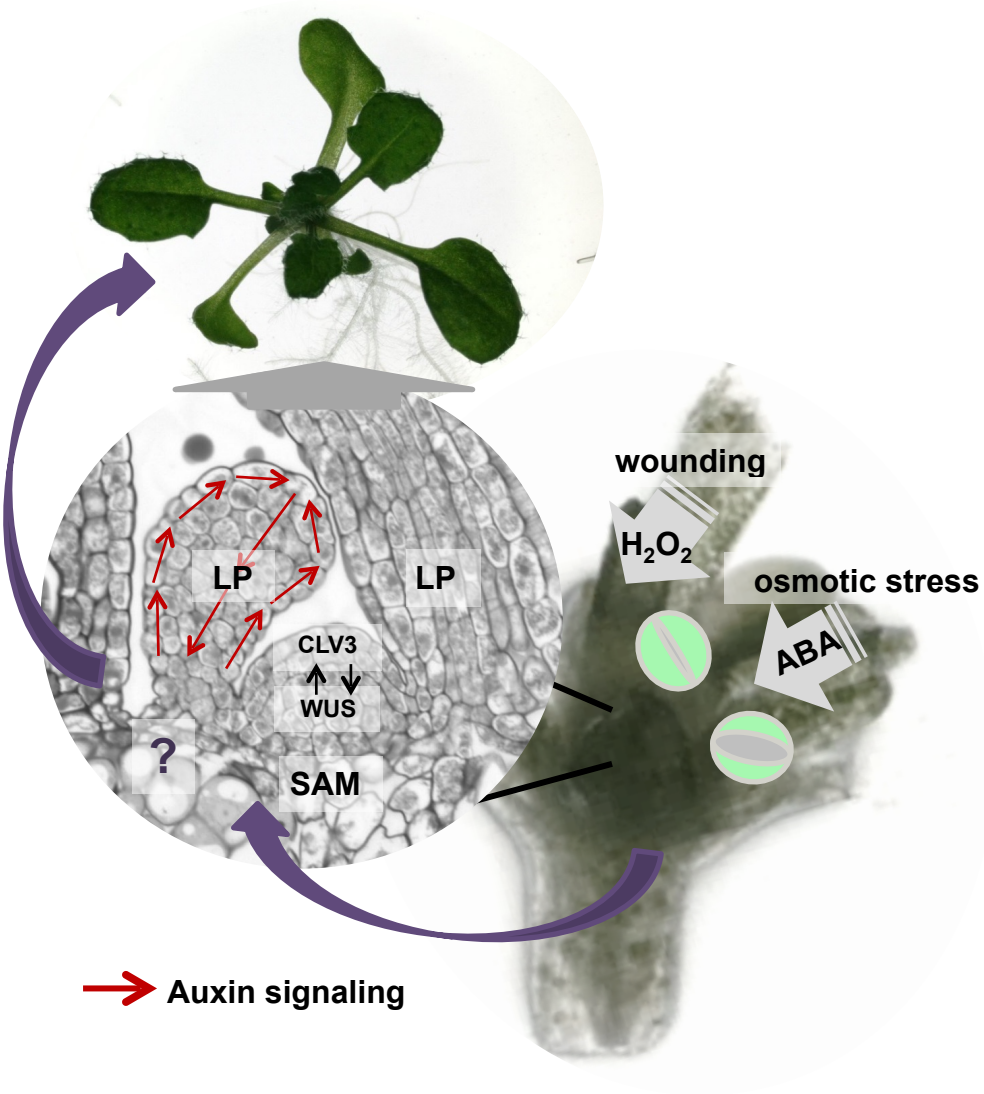



**Figure 40. Perception of cryostressors in the shoot apical meristem.** Cryostressors like wounding accompanied by $H_2O_2$, as well as osmotic changes regulated by stomatal aperture are perceived in shoot tips and affect SAM, which regulates post-cryogenic recovery of plantlets. LP: Leaf primordium; SAM: Shoot apical meristem; *CLV3*: *CLAVATA3*; *WUS*: *WUSCHEL*; ABA: abscisic acid.

## 4.6. Future perspectives

*Arabidopsis* appears to represent a suitable model for identifying the cryostress response imposed by cryopreservation. The conclusions reached from the present analysis should be relevant for the long-term storage of some important crop species (notably potato), for which a high rate of post-cryogenic recovery remains still confined to only a small number of germplasm accessions. Although high recovery in different WT genotypes restricts the possibility to find reliable genetic loci by quantitative mapping, the effect of silenced or overexpressed genes and TFs can be efficient shown in the wide range of available *Arabidopsis* mutants. A feasible approach to improve the success of cryopreservation could be the overexpression of proteins which provides advantages to important traits in cryopreservation like drought stress. Although recent studies showed that post-cryogenic recovery benefits from gene manipulation (El-Banna *et al.*, 2010), this strategy cannot be applied to wildtype gene bank accessions. Therefore, an applied approach to improve recovery of distinct gene bank accessions is the establishment of feeding experiments with phytohormones like ABA, SA or JA. In previous studies, the exogenous supply of Vitamin E and Vitamin C or GSH at critical steps of blackberry or citrus cryopreservation was applied (Uchendu *et al.*, 2010; Wang and Deng, 2004), and already resulted in significantly improved post-cryogenic recovery.

Beside new ideas for applied protocol improvement, this study was the first step to reveal the molecular mechanisms underlying cryostress. The *WRKY22*-dependent regulation of stomata closure emerged to be of importance during the cryoprotectant induced dehydration phase. Further experiments are necessary to understand the cross-communication of transcription factors of the *AP2-ERF* and *WRKY* family during this complex regulation machinery. Particularly the role of the *CBF*s during cryostress response is of certain interest. Additional investigation need to elucidate why the *CBF* transcripts showed lower abundance upon cryoprotection, although their expression have benefit effects for drought stress conditions.

Even though, *PR5* was induced in defense and drought stress response under the control of *WRKY22*, the biological function is still unclear.

Solving these question will help us to understand how plants respond to various types of abiotic stresses in nature.

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## CHAPTER 6. APPENDIX

Some parts of the appendix can only be found attached to the electronic version due to space limitation:

**Appendix: Data Set 1.** GO term enrichment

**Appendix: Data Set 2.** Hitlist

**Appendix: Data Set 3.** Mothertable

**Appendix Table 1**. Protocol for combined conventional and microwave-proceeded fixation, dehydration and resin embedding of *Arabidopsis* apical shoot meristem for histological and ultrastructural analysis.

**Microwave processing in a PELCO Bio Wave® 34700-230**

**(Ted Pella, Inc., Redding CA, USA)**

| Process | Reagent | | Power [W] | Time [sec] | Vacuum [mm Hg] |
|---|---|---|---|---|---|
| **1. Primary fixation** | 2.0 % (v/v) glutaraldehyde and<br>2.0 % (v/v) paraformaldehyde<br>in 0.05 M cacodylate buffer (pH 7.3) | | 150<br>0<br>150<br>0 | 60<br>60<br>60<br>60 | 0<br>0<br>0<br>0 |
| | | | + 12 h on shaker at RT | | |
| **2. Wash** | 1x 0.05 M cacodylate buffer (pH 7.3) and 2x aqua dest. | | 150 | 45 | 0 |
| **3. Secondary fixation** | 1 % (v/v) osmiumtetroxide in aqua dest. | | 0<br>80<br>0<br>80 | 60<br>120<br>60<br>120 | 10<br>10<br>10<br>10 |
| | | | + 15 min. on shaker at RT | | |
| **4. Wash** | 3x aqua dest. | | 150 | 45 | 0 |
| **5. Dehydration** | Acetone series: 20 %, 30 %, 40 %, 50 %, 60 %, 70 %, 80 %, 90 %, 2x 100 % | | 150 | 45 | 0 |
| | and 1 x propylen oxide | | +5 minutes each on shaker at RT | | |
| **6. Resin infiltration** | Spurr´s resin in propylen oxide | 25 %<br>50 %<br>75 %<br>100 % Spurr | 2 h on shaker at RT<br>2 h on shaker at RT<br>2 h on shaker at RT<br>12 h on shaker at RT | | |
| **7. Polymerisation** | 24 hrs at 70°C in Beem capsules in a heating cabinet. | | | | |

## APPENDIX

**Appendix Table 2a**. Wilcoxon Rank Sum Test followed Benjamini Hochberg correction among changes in transcript expression for MapMan Bins related to RNA-ProteinSynthesis in WT phase II over I (*P*<0.05).

| Bin | Name | Elements | p-value |
|---|---|---|---|
| **27.1** | **RNA.processing** | **379** | **< 1e-20** |
| 29.2.1 | protein.synthesis.ribosomal protein | 446 | < 1e-20 |
| 29.4 | protein.postranslational modification | 736 | 1.42E-19 |
| 29.1 | protein.aa activation | 94 | 3,15E-10 |
| 29.2.3 | protein.synthesis.initiation | 91 | 9,87E-10 |
| 29.2.4 | protein.synthesis.elongation | 34 | 2,42E-05 |
| 27.2 | RNA.transcription | 101 | 4,54E-04 |
| 29.2.2 | protein.synthesis.misc ribosomal protein | 14 | 2,81E-02 |
| 29.5 | protein.degradation | 2131 | 2,95E-02 |

**Appendix Table 2b**. Wilcoxon Rank Sum Test followed Benjamini Hochberg correction among changes in transcript expression for MapMan Bins related to RNA-ProteinSynthesis in WT phase III over II (*P*<0.05).

| Bin | Name | Elements | p-value |
|---|---|---|---|
| 27.1 | RNA.processing | 379 | 7.86E-8 |
| 29.5 | protein.degradation | 2131 | 3.63E-4 |

**Appendix Table 3**. The 33 most highly down-regulated genes in the WT explants identified in the contrast phase II *vs* I related to redox homeostasis. TPM: Transcript per million reads.

| | Locus | Phase I [TPM] | Phase II [TPM] | FC | Gene Name |
|---|---|---|---|---|---|
| 1 | AT1G20620 | 2707.73 | 96.17 | -4.8 | *Catalase 3* |
| 2 | AT1G19570 | 2292.88 | 616.10 | -4.7 | *Dehydroascorbate reductase* |
| 3 | AT1G11530 | 55.35 | 1.160 | -4.4 | *CXXS1* |
| 4 | AT5G18600 | 29.76 | 0.49 | -4.2 | *Thioredoxin superfamily protein* |
| 5 | AT4G25570 | 93.30 | 4.29 | -4.0 | *ACYB-2* |
| 6 | AT5G06690 | 44.20 | 1.81 | -3.7 | *WCRKC thioredoxin 1* |
| 7 | AT4G25100 | 226.20 | 16.20 | -3.4 | *Fe superoxide dismutase 1* |
| 8 | AT1G08570 | 62.60 | 4.83 | -3.3 | *CYS-HIS rich thioredoxin 4* |
| 9 | AT5G16400 | 37.68 | 2.87 | -3.0 | *Thioredoxin F2* |

| | | | | | |
|---|---|---|---|---|---|
| 10 | AT1G77490 | 33.67 | 3.31 | -2.8 | *Thylakoidal ascorbate peroxidase* |
| 11 | AT4G09010 | 151.24 | 20.53 | -2.8 | *Ascorbate peroxidase 4* |
| 12 | AT4G33040 | 28.56 | 3.30 | -2.5 | *Thioredoxin superfamily protein* |
| 13 | AT1G06650 | 51.72 | 8.53 | -2.4 | *2-oxoglutarat* |
| 14 | AT5G63030 | 94.27 | 16.88 | -2.3 | *Thioredoxin superfamily protein* |
| 15 | AT3G26060 | 262.49 | 51.75 | -2.2 | *Thioredoxin superfamily protein* |
| 16 | AT1G76080 | 28.96 | 5.54 | -2.2 | *Drought-induced stress protein* |
| 17 | AT2G47880 | 7.12 | 0.78 | -2.2 | *Glutaredoxin family protein* |
| 18 | AT5G08410 | 55.87 | 11.59 | -2.0 | *Ferredoxin/thioredoxin reductase subunit A* |
| 19 | AT1G03020 | 5.26 | 0.53 | -1.9 | *Thioredoxin superfamily protein* |
| 20 | AT4G03520 | 183.18 | 47.18 | -1.9 | *Thioredoxin superfamily protein* |
| 21 | AT1G03680 | 66.90 | 16.84 | -1.9 | *Thioredoxin M-type 1* |
| 22 | AT5G61440 | 4.86 | 0.66 | -1.8 | *CYS-HIS rich thioredoxin 5* |
| 23 | AT2G24940 | 93.76 | 26.03 | -1.8 | *Progesterone binding protein 2* |
| 24 | AT3G02730 | 39.40 | 10.64 | -1.8 | *Thioredoxin F-type 1* |
| 25 | AT5G21100 | 15.62 | 4.15 | -1.7 | *Plant L-ascorbate oxidase* |
| 26 | AT1G50950 | 9.52 | 2.31 | -1.7 | *Thioredoxin protein* |
| 27 | AT5G06290 | 67.71 | 21.58 | -1.6 | *2-cysteine peroxiredoxin B* |
| 28 | AT5G16710 | 85.14 | 27.44 | -1.6 | *Dehydroascorbate reductase 1* |
| 29 | AT4G29670 | 75.45 | 24.24 | -1.6 | *CYS-HIS rich thioredoxin 2* |
| 30 | AT5G06470 | 4.62 | 0.86 | -1.6 | *Glutaredoxin family protein* |
| 31 | AT5G18100 | 72.78 | 23.62 | -1.6 | *Copper/Zinc superoxide dismutase 3* |
| 32 | AT2G04700 | 92.46 | 30.23 | -1.6 | *Ferredoxin thioredoxin reductase* |
| 33 | AT1G14730 | 6.14 | 1.49 | -1.5 | *Cytochrome b561/ferric reductase transmembrane protein family* |

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## IV AFFIRMATION

Hereby, I declare that I have written the present dissertation independently (apart from the advice of my supervisors), without assistance from external parties and without use of other resources than those indicated. The ideas directly or indirectly from external sources are duly acknowledged in the text. The material, either in full or in part, has not been previously submitted for grading at this or any other academic institution.

## IV ERKLÄRUNG

Hiermit erkläre ich, dass ich die vorliegende Dissertation selbständig (abgesehen von der Beratung durch meine Betreuer), und unter ausschließlicher Verwendung der angegebenen Hilfsmittel verfasst habe. Wörtlich oder inhaltlich übernommene Stellen wurden als solche kenntlich gemacht. Diese Arbeit wurde bisher in gleicher oder ähnlicher Form noch keiner anderen Einrichtung zur Erlangung des Doktorgrades eingereicht. Diese Arbeit ist unter Einhaltung der Regeln guter wissenschaftlicher Praxis der Deutschen Forschungsgemeinschaft entstanden.

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## Publications and Reviews

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**Stock, J.,** Bräutigam, A., Melzer, M., Bunk, B., Nagel, M., Overmann, J., Keller, E.R.J. and Mock, H.-P. (2018). The defense-related transcription factor *WRKY22* is required during osmotic stress adaptation in *Arabidopsis* shoot tips. (*UNDER REVISION*)

**Stock. J.,** Mock, H.-P., Senula, A. and Nagel, M. (2018). *Arabidopsis* – A model to elucidate complex stress response mechanism during cryopreservation? (*IN PRESS*)

Bajerski, F., **Stock, J.,** Hanf, B., Darienko, T., Heine-Dobbernack, E., Eberth, S., Lorenz, M., Naujox, L., Keller, E.R.J., Schumacher, M., Mock, H.-P., Kniemeyer, T., Friedl, T. and Overmann, J. (2018). ATP Content and Cell Viability as Indicators for Cryostress Across the Diversity of Life. *Frontiers in Physiology* 9, 921.
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**Stock, J.,** Senula, A., Nagel, M., Mock, H.-P. and Keller, E.R.J. (2017). A Simple Method for Cryopreservation of Shoot Tips of *Arabidopsis* Genotypes. *CryoLetters* 38, 364-371.

Mustroph, A., **Stock, J.,** Hess, N., Aldous, S., Dreilich, A. and Grimm, B. (2013). Characterization of the phosphofructokinase gene family in rice and its expression under oxygen deficiency stress. *Frontiers Plant Science*, 4, 125.

**Poster Presentations (selected)**

---

**Stock, J.**, Bräutigam, A., Keller, E.R.J., Mock, H.-P. Regulatory Functions of *WRKY22* during *Arabidopsis* Cryopreservation. 30. Conference Molecular Biology of Plants, Dabringhausen, 21-24. February 2017.

**Stock, J.**, Petridis, A., Keller, E.R.J., Mock, H.-P**.** First Insights Into Cryostress Induced Stress Responses in *Arabidopsis* Shoot Tips. 29. Conference Molecular Biology of Plants, Dabringhausen, 23-26. February 2016.

**Stock. J.,** Senula, A. and Keller, E.R.J. DMSO Droplet Freezing and PVS2 Droplet Vitrification–Two methods of cryopreservation with differences in ice crystallization. Annual Scientific Meeting of the Society of Low Temperature Biology, Royal Kew Garden 2014

**Oral Presentations (selected)**

---

**Stock, J.**, Bräutigam, A., Keller, E.R.J., Nagel, M., Mock, H.-P. Defense-related *WRKY22* transcription factor mediates drought stress adaptation during *Arabidopsis* shoot tip cryopreservation. Botanikertagung, Kiel 2017

**Stock, J.**, Petridis, A., Nagel, M., Keller, E.R.J., Mock, H.-P. Cellular stress responses during cryo-induced stress in *Arabidopsis* shoot tips. CRYO2016 The 53rd Annual Meeting of the Society for Cryobiology, Ottawa 2016

**Stock, J.,** Petridis, A., Keller, E.R.J., Mock, H.-P. Identification of candidate genes related to the survival of *Arabidopsis thaliana* shoot tips after cryopreservation. CRYO 2015 The 52nd Annual Meeting of the Society for Cryobiology, Ostrava 2015

**Social Commitment**

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