UNIVERSIDADE ESTADUAL PAULISTA "JULIO DE MESQUITA FILHO"
FACULDADE DE CIÊNCIAS AGRONÔMICAS
CAMPUS DE BOTUCATU

RETENÇÃO DE CLOROFILA EM SEMENTES DE SOJA (*Glycine max* (L.) Merr.): ESTUDOS FISIOLÓGICOS, BIOQUÍMICOS E MOLECULARES

RENAKE NOGUEIRA TEIXEIRA

Tese apresentada à Faculdade de Ciências Agronômicas da UNESP – Campus de Botucatu, para obtenção do título de Doutor em Agronomia (Agricultura).

BOTUCATU-SP
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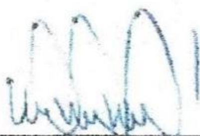

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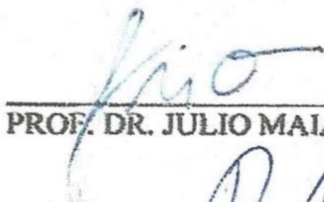

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CHLOROPHYLL RETENTION IN SOYBEAN SEEDS (*Glycine max* (L.) Merr.): PHYSIOLOGICAL, BIOCHEMICAL AND MOLECULAR STUDIES

RENAKE NOGUEIRA TEIXEIRA

Supervisor: Prof. PhD Edvaldo Aparecido Amaral da Silva

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"Learn from yesterday, live for today, hope for tomorrow. The important thing is not to stop questioning".

Albert Einstein

To my dear family, Vitor Hugo, Maria Helena and Diego.

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The plan at first was to make a list of names and acknowledgements following the "protocol", but a wise person told me I should write with my heart and let my personality be part of that, especially because, according to him the acknowledgments are the most read part of a thesis.

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Figure 23. Gene expression analysis by real-time quantitative PCR (RT-qPCR) represented as the calibrated normalized relative quantity (CNRQ) for PPH1, PPH2 and PPH3. Gene expression is shown for non-stressed (open bars) and stressed seeds (closed bars) of the susceptible (S) and tolerant (T) cultivars in three stages of maturation (R6-R7-R8). Bars represent the mean values ± SE of three independent biological replicates. Bars connected by brackets represent the statistical comparisons. Asterisks represent statistically significant differences (*, P ≤ 0.05; **, P ≤ 0.01). 60

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(closed bars) of the susceptible (S) and tolerant (T) cultivars in three stages of maturation (R6-R7-R8). Bars represent the mean values ± SE of three independent biological replicates. Bars connected by brackets represent the statistical comparisons. Asterisks represent statistically significant differences (*, P ≤ 0.05; **, P ≤ 0.01). 62

Figure 25. Gene expression analysis by real-time quantitative PCR (RT-qPCR) represented as the calibrated normalized relative quantity (CNRQ) for *RCCR1* and *RCCR2*. Gene expression is shown for non-stressed (open bars) and stressed seeds (closed bars) of the susceptible (S) and tolerant (T) cultivars in three stages of maturation (R6-R7-R8). Bars represent the mean values ± SE of three independent biological replicates. Bars connected by brackets represent the statistical comparisons. Asterisks represent statistically significant differences (*, P ≤ 0.05; **, P ≤ 0.01). 63

Figure 26. Gene expression analysis by real-time quantitative PCR (RT-qPCR) represented as the calibrated normalized relative quantity (CNRQ) for *D1* and *D2*. Gene expression is shown for non-stressed (open bars) and stressed seeds (closed bars) of the susceptible (S) and tolerant (T) cultivars in three stages of maturation (R6-R7-R8). Bars represent the mean values ± SE of three independent biological replicates. Bars connected by brackets represent the statistical comparisons. Asterisks represent statistically significant differences (*, P ≤ 0.05; **, P ≤ 0.01). 65

Figure 27. Gene expression analysis by real-time quantitative PCR (RT-qPCR) represented as the calibrated normalized relative quantity (CNRQ) for *Cyt* B_6F and *LCHA*. Gene expression is shown for non-stressed (open bars) and stressed seeds (closed bars) of the susceptible (S) and tolerant (T) cultivars in three stages of maturation (R6-R7-R8). Bars represent the mean values ± SE of three independent biological replicates. Bars connected by brackets represent the statistical comparisons. Asterisks represent statistically significant differences (*, P ≤ 0.05; **, P ≤ 0.01). 67

Figure 28. Gene expression analysis by real-time quantitative PCR (RT-qPCR) represented as the calibrated normalized relative quantity (CNRQ) for *psbB* and *pcbC*. Gene expression is shown for non-stressed (open bars) and stressed seeds (closed bars) of the susceptible (S) and tolerant (T) cultivars in three stages of maturation (R6-R7-R8). Bars represent the mean values ± SE of three independent biological replicates. Bars connected by brackets represent the statistical comparisons. Asterisks represents statistically significant differences (*, P ≤ 0.05; **, P ≤ 0.01). 69

Figure 29. Gene expression analysis by real-time quantitative PCR (RT-qPCR) represented as the calibrated normalized relative quantity (CNRQ) for *psbA* and *psbD*. Gene

expression is shown for non-stressed (open bars) and stressed seeds (closed bars) of the susceptible (S) and tolerant (T) cultivars in three stages of maturation (R6-R7-R8). Bars represent the mean values ± SE of three independent biological replicates. Bars connected by brackets represent the statistical comparisons. Asterisks represents statistically significant differences (*, P ≤ 0.05; **, P ≤ 0.01). 70

Figure 30. Gene expression analysis by real-time quantitative PCR (RT-qPCR) represented as the calibrated normalized relative quantity (CNRQ) for *psaA* and *psaB*. Gene expression is shown for non-stressed (open bars) and stressed seeds (closed bars) of the susceptible (S) and tolerant (T) cultivars in three stages of maturation (R6-R7-R8). Bars represent the mean values ± SE of three independent biological replicates. Bars connected by brackets represent the statistical comparisons. Asterisks represents statistically significant differences (*, P ≤ 0.05; **, P ≤ 0.01). 73

ABREVIATIONS

Chl – chlorophyll

CLH – chlorophyllase

NYC – non-yellow coloring

NOL – non-yellow coloring like

PPH – pheophytinase

PAO – pheophorbide a oxigenase

RCC – red chlorophyll catabolite

RCCR – red chlorophyll catabolite reductase

pFCC – primary fluorescent chlorophyll catabolite

NCC – non-fluorescent chlorophyll catabolite

phein – pheophytin

pheide – pheophorbide

LHC – light harvesting complex

HCAR – hydroxyl-methyl chlorophyll a reductase

SGR – stay-green

CCE – chlorophyll catabolic enzymes

HPLC – high performance liquid chromatography

CNRQ – calibrated normalized relative quantity

ORA – over representation analysis

GO – gene ontology

PSI – photosystem I

PSII – photosystem II

DP – dominant pattern

ABSTRACT

Brazil is the second largest soybean producer in the world and most of its production is located in the tropical area of the country which is prone to drought and high temperature. Under such stressful conditions it is common to observe the retention of chlorophyll at the end of seed maturation. Very little is known about the role of chlorophyll in developing seeds, but its presence in oilseeds enhances oxidation of the extracted oil, resulting in reduced shelf life, unappealing appearance and a lower price for the producer. Also seed quality for propagation is severely affected by chlorophyll retention. Besides environmental conditions, there are also genetic components controlling the sensitivity of different cultivars to retain chlorophyll. A detailed characterization of green seed production by two different cultivars under stressed conditions and its effect on seed lot quality was carried out. In an attempt to determine the molecular basis of chlorophyll degradation and, consequently, its retention, a transcriptome analysis was performed using stressed (green) and non-stressed (yellow) soybean seeds at three stages of maturation: R6, R7 and R8. Multiple ontologies were enriched in soybean seeds produced under stress. Among the top-ranking was the photosynthetic process, particularly the photosystem (PS) II category. The expression analysis suggests higher levels of the proteins D1 (psbA), D2 (psbD), A1 (psaA) and A2 (psaB) core subunits of PSII and PSI, in stressed/green seeds. Special attention was also given to chlorophyll catabolic enzymes and to stay-green genes, among which, disturbances in expression of *PPH2*, *D1* and *D2* caused by environmental stresses appears to be the major cause for chlorophyll retention in soybean seeds.

Keywords: Gene expression, green seeds, seed quality, soybean, stress

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RESUMO

O Brasil é o segundo maior produtor de soja do mundo e maior parte da produção se encontra nas áreas tropicais do país, mais propensas à ocorrência de seca e de altas temperaturas. Sob tais condições estressantes é comum a ocorrência de retenção de clorofila nas sementes ao final da maturação. Muito pouco se sabe a respeito do papel da clorofila em sementes em desenvolvimento, mas a presença de tal pigmento em sementes oleaginosas aumenta a oxidação do óleo extraído, resultando em redução na vida de prateleira, aparência desagradável e consequentemente preços mais baixos pagos ao produtor. A qualidade fisiológica das sementes também é severamente afetada. Além das condições ambientais, fatores genéticos também controlam a susceptibilidade à retenção de clorofila. Caracterização detalhada da produção de sementes verdes por duas cultivares conduzidas sob condições de estresse e os efeitos sobre a qualidade fisiológica das sementes foi realizada. Na tentativa de determinar os mecanismos moleculares da degradação de clorofila em sementes e consequentemente sua retenção, uma análise de transcriptoma foi conduzida utilizando-se sementes estressadas (verdes) e não estressadas (amarelas) de soja em três estádios de maturação; R6, R7 and R8. Múltiplas ontologias foram significativamente afetadas pelo estresse. Entre as mais alteradas estão os processos fotossintéticos, particularmente a categoria fotossistema II. A análise de expressão gênica sugere aumento na expressão das proteínas D1 (psbA), D2 (psbD), A1 (psaA) e A2 (psaB), subunidades principais dos fotossistemas II e I, em sementes estressadas/verdes. Atenção especial também foi dada a enzimas do catabolismo de clorofila e a genes stay-green, dentre os quais, distúrbios na expressão de *PPH2*, *D1* e *D2* podem ser a principal causa da retenção de clorofila em sementes de soja.

Palavras-chave: Expressão gênica, sementes verdes, qualidade de sementes, estresse.

1. Introduction

Chlorophyll (Chl) retention in mature soybean seeds is frequently reported by Brazilian soybean growers as an undesirable trait, since the presence of green seeds in a lot is a downgrading factor for marketing. The "green seed problem" has been reported in oilseeds since early 1980s, i.e. as detrimental to canola oil quality (JOHNSON-FLANAGAN; THIAGARAJAH, 1990; GREEN et al., 1998; MAILER et al., 2003; CHUNG et al., 2006) as well as a problem for soybean seed and oil quality (COSTA et al.,2001; GOMES et al., 20003; PÁDUA et al., 2007; PÁDUA;FRANÇA-NETO; et al., 2009; ZORATO et al., 2007).

Retention of Chl in soybean seeds is mainly associated with low rainfall and high temperatures during the maturation phase. These are common environmental conditions in tropical areas such as the Brazilian cerrado where most of the Brazilian soybean seed production is concentrated. Besides the climate, other factors or practices during pre- and post-harvesting such as application of desiccants and premature harvesting followed by drying at high temperatures are also reported as causal for retention of the green pigment. These unfavorable climate conditions, agricultural practices, and post-harvest treatments may cause impairments in Chl degradation and the Chl degradation pathway may be also differently affected depending on the genetic material.

The green pigment is the key component for photosynthesis. It exists bound to proteins of the photosystems, but mainly to light-harvesting complexes I (LHCI) and II (LHCII). During leaf senescence, the most apparent observable change is

yellowing of green tissues. The same phenomenon is observed during maturation as degreening of the seeds, although maturing seeds are not in a senescence process. The loss of green color is caused by Chl degradation and breakdown of LHCI and LHCII and probably of the other photosynthetic Chl-proteins as well.

The first step in Chl degradation is the conversion of Chl b to Chl a, catalyzed by Chl b reductase. Subsequently, Chl a is degraded to a colorless, blue-fluorescing intermediate (pFCC) by a series of enzymes, such as pheophytinase (PPH), pheophorbide a oxygenase (PAO), and red chlorophyll catabolite reductase (RCCR). Thereafter, pFCC is transferred to the cytosol and stored in vacuoles as a non-fluorescent Chl catabolite (NCC) (ECKHARDT et al., 2004; HÖRTENSTEINER, 2006, 2013; SATO et al., 2009; HÖRTENSTEINER; KRÄUTLER, 2011; SAKURABA et al., 2012).

Under some conditions Chl breakdown can be delayed or inhibited, resulting in delayed yellowing, called ‘stay-green’ phenotype. Different routes leading to this phenotype were described by Thomas and Howarth (2000). Many mutants have been described as stay-green genotypes in different plant species, displaying Chl retention in leaves, pods or seeds (GUIAMÉT et al., 1991; CLERKX et al., 2003; TANAKA et al., 2003; PRUZINSKÁ et al., 2003; PARK et al., 2007; REN et al., 2007; SATO et al., 2007, 2009; WU et al., 2007; SCHELBERT et al., 2009; HORIE et al., 2009; YAMATANI et al., 2013). Although chlorophyll retention in soybean seeds is a problem caused by environmental factors, it occurs in variable intensity depending on the genetic material and it could also be treated as a “mild stay green phenotype”.

Chl content of mature seeds was a trait studied to determine breeding lines of Australian canola (MAILER et al., 2003). But, there is no report on this trait as part of the soybean breeding programs.

Abnormal weather has played a huge role in the sharp decline of soybean yields and it has also been related to the “green seed problem”. Efforts have already been made to understand the influence of growing conditions and drying processes on the retention of green pigments in the seeds and how it affects the physiological and biochemical characteristics of these seeds. So far, the molecular mechanisms of Chl degradation in seeds and consequently of Chl retention remain unsolved.

The aim of this study was to evaluate the influence of heat and drought stress on chlorophyll retention in soybean seeds and the expression of genes related to this phenomenon. A gene expression study was performed to elucidate the

molecular basis of the Chl degradation in stressed soybean seeds, namely the "green seed problem". In addition to that, a detailed characterization of green seed production in two different genotypes under stressed condition and its effect on seed lot quality was performed.

2. Review

2.1. The “green seed problem”

Brazil is the second largest soybean producer in the world. In 2014 the Brazilian production had an increase of 5.6% in relation to 2013. A total of 86,052.2 tons were harvested from 30,105.4 hectares. This increase was mainly due to an increase in sowed area as the yield was reduced in some regions. For example in the South and Southeast of the country there was a reduction of around 7% in the yield due to climate adversities (CONAB, 2014).

For some years Brazilian soybean growers have reported the occurrence of green seeds at the end of the maturation process. The green color of the seeds is a result of the presence of high amount of Chls and their derivatives. This phenomenon is generally associated with climate adversities during the ripening stage which lead to a rapid decrease in water content and impairment of the natural degreening.

During early seed development the soybean embryo is green. Under normal environmental conditions on the field, Chl is completely degraded during seed maturation resulting in a yellow seed. Normal seed degreening appears to take place via a controlled process of plastid degradation; with changes in chloroplast ultrastructure and degradation of Chl-protein complexes occurring as the seed moisture content decreases during the pre-desiccation stage (JOHNSON-FLANAGAN; SPENCER, 1996). This catabolism can be understood as a detoxifying process for the plant due to the loss of photodynamic and pro-oxidant properties of this pigment (PRUZINSKÁ et al., 2003; ECKHARDT et al., 2004).

In general, degreening is blocked by high temperatures and drought. Therefore, several factors occurring pre- and post-harvesting can lead to the retention of the green pigment, such as severe rust that causes the premature death of the plants, application of desiccants to anticipate harvesting and premature harvesting followed by fast drying at high temperatures (GOMES et al., 2003). These unfavorable climate conditions, agricultural practices and post-harvest treatments affect the normal flow of the degradation pathway of Chl.

Although the environment has the major effect on Chl retention, it is also influenced by genetic components. Different genetic materials have been shown to present variable susceptibility to Chl retention in canola (WARD et al., 1992; MAILER et al., 2003) and in soybean (PÁDUA; CARVALHO; et al., 2009).

The presence of Chl in oilseeds such as canola and soybean is considered a downgrading factor since it is one of the parameters used for quality control of these seeds, both for the oil industry and for propagation purposes ((WARD et al., 1995; COSTA et al., 2001; DAUN, 2003; ZORATO et al., 2007; CICERO et al., 2009; PÁDUA; FRANÇA-NETO; et al., 2009).

The Chl and most of its derivatives are lipid-soluble compounds and therefore are extracted with the oil when green seeds are crushed resulting in the production of dark-colored crude oil, which is commercially unacceptable and may require additional bleaching steps to remove these pigments and, therefore, raising the production costs significantly (TAUTORUS; LOW, 1993). When present in oils, Chl pigments act as photosensitizers, reducing its oxidative stability, which may lead to rancidity, and reduced shelf life (MINGUEZ-MOSQUERA et al., 1990; TAUTORUS; LOW, 1993).

There is also a negative correlation between the retention of Chl and seed quality for propagation. It is suggested that hot and dry weather conditions may cause forced maturation of the soybean seed, resulting in high levels of green seeds in a seed lot. The green seeds are smaller and lighter showing higher levels of leachate, faster rate of deterioration and produce shorter seedlings. Thus the presence of Chl in soybean seeds is commonly associated with lower viability, germination, vigor and longevity (COSTA et al., 2001; ZORATO et al., 2007; CICERO et al., 2009; PÁDUA; FRANÇA-NETO; et al., 2009). Similarly, Chl retention in seeds of *Arabidopsis thaliana* (green-seeded) mutants causes a decrease in seed viability, vigor and longevity (CLERKX et al.,

2003; NAKAJIMA et al., 2012). The effect of Chl retention on the physiological quality of the seed lot is dependent on the percentage of green seeds present in this lot.

Various classification systems have been used to control and standardize the production of soybean seeds for international marketing. Each country has set rules regarding trading procedures, grades and standards. The various criteria used to evaluate soybean quality are based on several physical characteristics as damaged kernels, splits, other colors than yellow and foreign matter (GOMES et al., 2003). Attempts have been made to determine safe levels of green seeds in a seed lot regarding its physiological quality for propagation. Costa et al. (2001) suggest a maximum of 10% of green soybean seeds in a seed lot while Pádua et al. (2007) mention a limit of 9% of green seeds.

Different countries have different levels of tolerance and grade standards differ in their maximum percent limits (SINCLAIR, 1995; LIU, 2000). In Brazil, the parameters for soybean are based on moisture content, damaged kernels, splits, impurities, and the presence of green-colored seeds. The Brazilian grade standards are more tolerant than those in international trade concerning the allowed proportion of green seeds (BORRMANN et al., 2009). While the threshold for off-color soybeans is 1% in the USA, the maximum permitted level of greenish seeds for marketing in the Brazilian legislation is 8% (BRASIL, 2007).

As discussed above, the presence of Chl in mature seeds has not only negative effects on oil quality, but also on the physiological quality of the seeds for propagation. However, still little is known about the role of Chl in oilseeds or the mechanisms of degreening during seed maturation.

2.2. The occurrence and role of Chl pigments in developing and maturing seeds

Several of the major oilseed crops (e.g. soybean, rapeseed, cotton, and linseed) produce seeds that are green during their development (RUUSKA et al., 2004). In fact, the presence of Chl in seeds was described as early as 1909 (MONTEVERDE; LYUBIMENKO, 1909; YAKOVLEV; ZHUKOVA, 1980). Chl content has been measured in mature seed embryos of 1094 plant species from 182 families of angiosperm plants and it was concluded that this pigment is present in seeds of 428 species from 72 families (SMOLIKOVA et al., 2011). Although the seeds are predominantly sink

tissues, the green ones contain chloroplasts with the thylakoid structures and enzymes of typical photosynthetic machinery. This fact has prompted questions regarding the mechanisms and contributions of seed photosynthesis to oilseed metabolism. Despite the well-known fact of Chl presence in seeds, biological significance of this phenomenon remains largely unresolved to date and very few studies have been devoted to elucidate it.

Schwender et al. (2004) states that even the decreased amount of light that penetrates the fruit wall of *Brassica napus* L. can provide reducing power and ATP that may expand the range of pathways available for storage product synthesis in seeds. In maturing seeds, Chl appears to function in maintaining photosynthetic capacity (BORISJUK et al., 2004; WEBER et al., 2005), although photosynthetic CO_2 fixation is low in embryos compared with that in leaves (ASOKANTHAN et al., 1997).

The photosynthetic energy transfer occurring within the seed of *Brassica napus* was assessed by the measurement of the linear electron transport rate (ETR). Both the testa and the outer cotyledon displayed substantially higher levels of ETR during the main storage phase compared with the inner embryo region. When exposed to close to saturating light levels, the ETR was by far the highest in the testa and in the inner cotyledon only 67% of that of the outer cotyledon. This gradient in photosynthetic performance is even fortified in planta by the gradient in light supply from the outside to the inside of the seed (BORISJUK et al., 2013).

Nevertheless, photosynthesis in embryos is not essential for seed development, as was demonstrated in Chl-deficient seeds of the *Arabidopsis thaliana* albino mutant which were able to germinate (SUNDBERG et al., 1997). Thus, it seems that seed photosynthesis plays additional roles above CO_2 fixation in developing seeds (ALBRECHT et al., 2008). It has been suggested that the main function of seed photosynthesis is to increase the O_2 concentration in developing seeds in order to sustain ATP synthesis in mitochondria (BAUD; LEPINIEC, 2010). The demand for ATP synthesis appears to be high in oilseeds in which large amounts of lipids are synthesized. There is evidence that developing embryos are photosynthetic active, and this process seems to be directly associated with fatty acid biosynthesis of the developing seed (WILLMS et al., 1999; RUUSKA et al., 2004). In addition to that, in developing seeds of *Brassica napus* the application of additional O_2 enhanced ATP levels and lipid biosynthesis (VIGEOLAS et al., 2003).

Rather than producing carbon to export as in source tissues, photosynthesis in seeds could participate in metabolism in at least three ways according to Ruuska et al. (2004). First, light reactions could produce NADPH and ATP for energetically expensive fatty acid synthesis; second, O_2 production may help to prevent anoxia inside seeds (ROLLETSCHEK et al., 2002); and third, the dark reactions of photosynthesis could improve the biosynthetic efficiency of seeds by refixing respiratory CO_2 and providing intermediates for metabolism.

Thus, although it is not essential, it is feasible that photosynthesis plays a role in seed development. Despite the available information about photosynthesis in seeds and the importance of Chl to this process, the function and regulation of Chl degradation during the late phase of seed maturation are not well understood.

It is widely known that Chl degradation plays a role in leaf senescence when nutrients, in particular nitrogen and phosphorus, are remobilized to sink organs, such as storage tissues or seeds. Therefore, Chl degradation during seed maturation cannot be associated with senescence and it is possible that Chl degradation has additional physiological functions in seeds compared with those in leaves.

As mentioned previously, Chl content has been reported to be inversely related to seed quality. However, it is not known if there is a direct effect of Chl on seed quality. It remains unknown whether the Chl is the cause of the low quality or if it is a marker of disturbances during maturation. It is common knowledge that seed quality is acquired during seed maturation, e.g. by the synthesis of specific stress related proteins, such as the late embryogenesis abundant (LEA) proteins and heat shock proteins (HSPs). Concomitantly, seed Chl is degraded (BEWLEY et al., 2013). Thus, high Chl levels in seeds are usually linked with immaturity and, hence, low seed quality, independently of a possible negative effect of Chl pigments. In fact, Chl fluorescence is commonly used as a sorting tool in Brassica ssp. to eliminate the immature high-Chl seed fractions of low quality (JALINK et al., 1998). In other words, Chl content is, besides being a marker for the progress of seed maturation is also a signal for stress during seed maturation as mentioned above.

2.3. Chlorophyll degradation

Chl is the most abundant pigment on earth. It is essential for light absorption during photosynthesis. Higher plants contain two species of Chl, namely Chl a and Chl b. The Chl a contains a methyl group at position 7 on the tetrapyrrole macrocycle, while the Chl b contains a formyl group at the same position (Figure 1). Chl a is the major Chl and resides both in the core complexes of photosystem (PS) I and II, and in the peripheral antenna complexes, which are composed of light-harvesting Chl-binding proteins (SATO et al., 2009).

Chl a not only harvests and transfers light energy, but also play a role in photochemistry and electron transfer. In contrast, Chl b is exclusively associated with the peripheral antenna complexes in plants. The major function of Chl b is harvesting and transferring light energy but this function is also a property of Chl a. As a result Chl b is not essential to plant photosynthesis. Nevertheless, plants need Chl b to stabilize light harvesting complex (LHC) (TANAKA; TANAKA, 2011; NICKELSEN; RENGSTL, 2013; KUSABA et al., 2013). Chl b is synthesized from Chl a and is catabolized only after it is reconverted to Chl a. This inter-conversion system between Chl a and Chl b is referred to as the Chl cycle (TANAKA; TANAKA, 2011).

The Chl a, together with apoproteins of the photosynthetic apparatus, is degraded during senescence, fruit ripening or seed degreening/maturation of most species. It remains unknown whether the mechanism of Chl breakdown under all these conditions is the same. Very little is known about the degradation of this pigment in seeds and most of the studies regarding Chl breakdown are done with help of stay-green mutants and induced leaf senescence, as will be discussed in this review.

The loss of green pigment is the first visible change that occurs during senescence and this event is associated with the breakdown of photosynthetic proteins. To initiate Chl degradation, Chl b is first converted to Chl a, and then all Chl molecules seem to follow the same degradation pathway. Thus conversion of Chl b to Chl a is a prerequisite for Chl breakdown and it is carried out in two reductive steps with 7-hydroxymethyl Chl a as an intermediate.

In the first step, the 7-formyl group of Chl b is reduced to a hydroxymethyl group by Chl b reductase (KUSABA et al., 2007). This enzyme is an NADPH-dependent short-chain dehydrogenase. All plants examined so far have two types

of Chl b reductase (NYC1 or NOL) (KUSABA et al., 2013). NYC1 possesses putative membrane-spanning regions, while NOL does not. In rice, NON-YELLOW COLORING1 (NYC1) and NYC1-LIKE (NOL) seem to form a hetero dimer (SATO et al., 2009), while in Arabidopsis they appear to function independently (Horie et al. 2009). The second step of the chl b to chl a conversion is carried out by 7-hydroxymethyl chl a reductase (HCAR), which is an Fe–S containing flavoprotein. This enzyme reduces 7-hydroxymethyl-Chl into Chl a using ferredoxin as a reductant (HÖRTENSTEINER, 2013; KUSABA et al., 2013).

The first step of Chl a breakdown involves the removal of Mg^{2+} to form pheophytin a (phein a). PPH, a hydrolase belonging to the serine protease family, then catalyzes the removal of the phytol residues from phein a to form pheophorbide a (pheide a) (SCHELBERT et al., 2009; MORITA et al., 2009). Until recently, uncertainty existed about the order of these two reactions, with phytol removal preceding Mg-chelation being the favored hypothesis (HÖRTENSTEINER, 2006; TANAKA; TANAKA, 2006). In agreement with this, chlorophyllase, which hydrolyzes phytol from Chl, was believed to be active during senescence-related Chl breakdown (TAKAMIYA et al., 2000). However, so far the chlorophyllase action has been stated as essential or not essential depending on the species. Nevertheless, there is compelling evidence that during leaf senescence, at least in Arabidopsis, dephytylation occurs after Mg removal, by the phein-specific action of PPH. The mechanism of Mg-dechelation has not been resolved in detail until now (HÖRTENSTEINER, 2013).

Downstream of the Chl breakdown, pheide a, a genuine intermediate of the pathway (LANGMEIER et al., 1993; VICENTINI et al., 1995), has its macrocycle ring opened by pheophorbide a oxygenase (PAO), a Rieske-type 2Fe–2S oxygenase, to produce red Chl catabolites (RCC) (TANAKA et al., 2003; PRUZINSKÁ et al., 2003).

In the next step the C20/C1 double bond of RCC is reduced to form primary fluorescent Chl catabolite (pFCC) by red Chl catabolite reductase (RCCR) (PRUŽINSKÁ et al., 2005). This enzyme belongs to the ferredoxin-dependent bilin reductase family (SUGISHIMA et al., 2009). The reaction can give rise to two possible C1-stereoisomers, pFCC or epi-pFCC (HÖRTENSTEINER et al., 2000; PRUŽINSKÁ et al., 2007). Downstream of pFCC or epi-pFCC there is a diversity of fluorescent Chl catabolites (FCCs) and non-fluorescent Chl catabolites (NCCs), indicating that many different enzymatic reactions occur. The NCCs are colorless linear tetrapyrroles, final

products of the Chl breakdown and to date more than 15 different NCCs have been identified from over 10 plant species. All these NCCs have an identical open tetrapyrrole backbone (HÖRTENSTEINER, 2006; HÖRTENSTEINER; KRÄUTLER, 2011).

The early reactions of the pathway take place in the plastid and end with the formation of a primary fluorescent Chl catabolite (pFCC). After export from the plastid, several peripheral side chains of pFCC are modified in the cytosol to produce a species-specific variety of FCCs. Finally, after import into the vacuole, these modified FCCs are isomerized to their respective NCCs in a non-enzymatic reaction driven by the acidic vacuolar pH (OBERHUBER et al., 2003). Thus, based on the types of modifications to FCC, different plant species accumulate a characteristic set of NCCs during senescence (HÖRTENSTEINER, 2009).

Pigment color changes as the catabolic pathway proceeds. Chl a is green and Chl b appears slightly lighter in color when the pigments are dissolved in organic solvents. The color of these pigments are somewhat modified when they are bound to proteins within the cells. Phein a and pheide a appear a dull green and RCC is red. pFCC and the downstream catabolites are all colorless in the visible light spectrum (KUSABA et al., 2013). Figure 1 illustrates the Chl degradation pathway described here.

In summary, the plastid-located part of the Chl degradation pathway starts with the removal of the central Mg atom by a metal chelating substance, whose molecular nature is yet unknown, originating phein a as an intermediate, and is followed by phytol hydrolysis yielding pheide a. Next, pheide a is converted to RCC by PAO and is then reduced to pFCC in a regio- and stereoselective manner catalyzed by RCCR.

At the same time, the breakdown of Chl seems to require an extra protein. Screening for stay-green mutants in many species uncovered a novel chloroplast-located protein, termed STAY-GREEN (SGR) (HÖRTENSTEINER, 2009), the function of which is considered to be related to Chl breakdown, but not an enzyme of the degradation pathway itself. During active Chl breakdown, dynamic SGR-CCE-LHCII (stay-green, Chl catabolic enzymes, light harvesting complex II) protein interaction occurs at the thylakoid membrane. The likely role of these interactions is to metabolically channel Chl breakdown pigments to minimize the risk of photoxidation of these light-excitable intermediates and, thus, to prevent accelerated cell death during leaf senescence (SAKURABA et al., 2012).

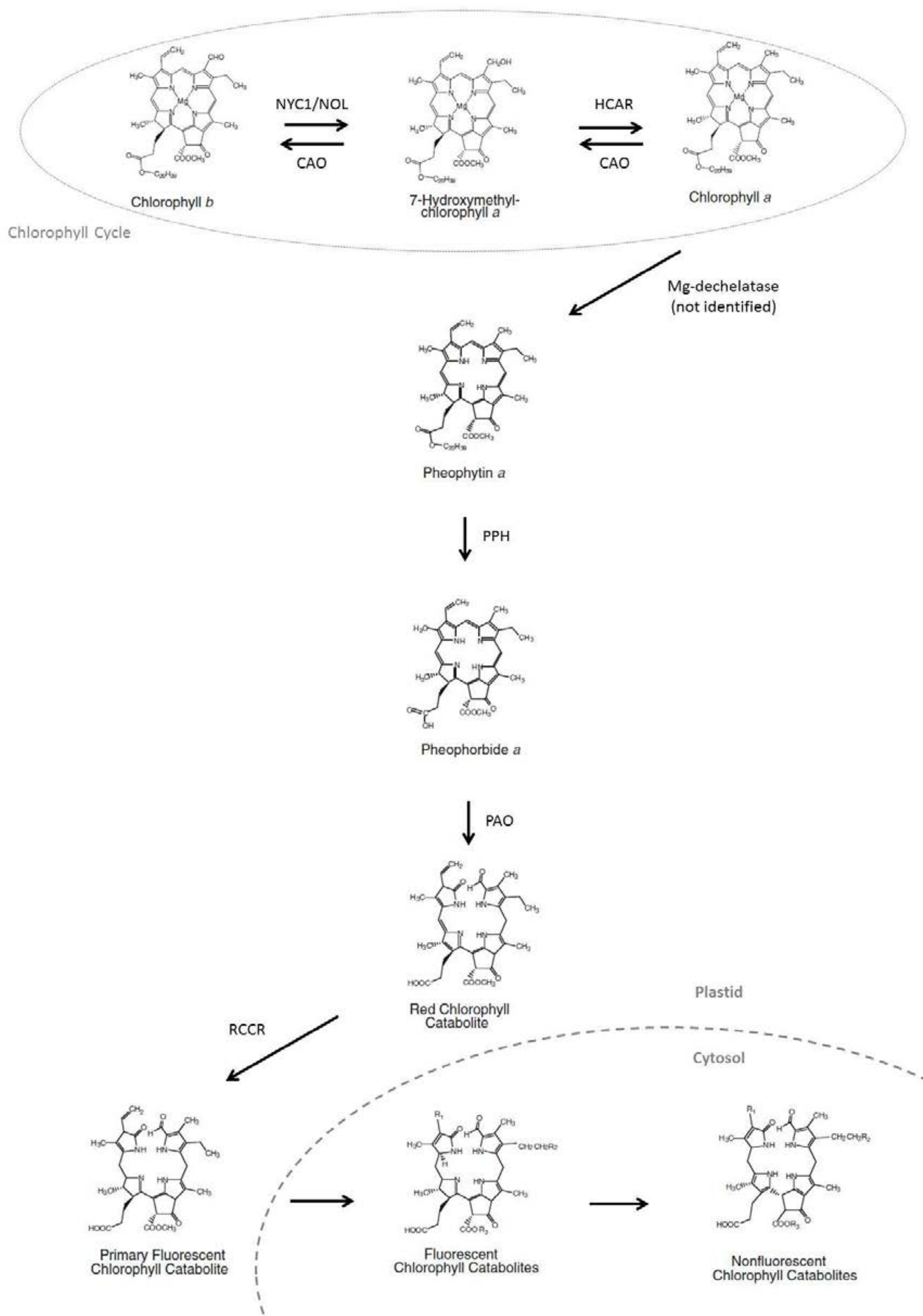


Figure 1. Chl a and b molecules and their breakdown pathway. The chemical structures are based on molecules identified in Arabidopsis (KUSABA et al., 2013). R1 denotes ethyl, while R3 can be either H or a methyl group. In the structure of FCC, R2 can be either OH or H, while for NCC, R2 is H, OH or O-glucosyl. The

side chains of FCC and NCC vary in between species (HÖRTENSTEINER; KRÄUTLER, 2011). The Chl cycle is explained in detail in Tanaka and Tanaka (2011). CAO=Chl a oxigenase, HCAR=hydroxymethyl-Chl a reductase, NOL=NYC1-like, NYC1=non-yellow coloring1, PPH=pheophytinase, PAO=pheide a oxygenase, RCCR=red Chl catabolite reductase.

2.4. The stay-green phenotype

Chl breakdown is part of the leaf senescence program, genetically determined and highly ordered and leads to the green color loss and, more importantly, in leaves it remobilizes nutrients, in particular nitrogen and phosphorus, to sink organs, such as storage tissues or seeds. Initiation and progression of this process is executed by a complex regulatory network with numerous specific genes and proteins involved (SATO et al., 2009; KUSABA et al., 2013). Although many of these genes and proteins, including transcriptional regulators have been identified, the understanding of leaf senescence, and thereby Chl degradation, especially in seeds, is still limited.

Impairments in the Chl breakdown seem to have two possible consequences: (1) accumulation of phototoxic intermediates of the pathway leading to accelerated cell death (by oxidative damage) or, (2) Chl retention in leaves or seeds. Mutants, varieties or transgenic lines of plants that retain the green color are known as 'stay-green' plants. There are mutants among different species, including many crops that retain greenness of leaves during senescence and also, some that retain Chl in the seeds.

There are several different routes to the stay-green phenotype in leaves. The major characteristics that classify the different kinds of stay-green are the initiation of Chl degradation, the rate of degradation and the photosynthetic activity. According to the classification proposed by Thomas and Howarth (2000), the stay-green plants can be separated in five types. Type A delays degradation initiation, but maintains a normal rate; type B has normal initiation but at a slower degradation rate; type C has both normal initiation and degradation rate, but with a lesion in Chl degradation; while type E also has normal initiation and degradation rate, but enhanced greenness. In some situations, Chl breakdown can be inhibited by rapid tissue death, referred to as type D.

Stay-green mutants in the five categories described above can be further divided in two main categories: (1) functional and (2) non-functional (also termed

cosmetic) mutants. In the functional stay-green mutants there is maintenance of photosynthetic activity while in the cosmetic stay-green mutants there is loss of the photosynthetic activity. Functional stay-green mutants could be affected in timing of senescence initiation or speed of senescence progression (HÖRTENSTEINER, 2009). Agronomically, functional stay-green mutants are interesting because delaying of senescence initiation (type A) or progression (type B) might have an advantageous effect on yield (THOMAS; HOWARTH, 2000).

Many stay-green plants have been described, with different gene products being responsible for the green phenotype. For example *Oriza sativa* stay-green mutants *nyc1* (KUSABA et al., 2007), *nol* (SATO et al., 2009), *sgr* (JIANG et al., 2007; PARK et al., 2007), *nyc4* (YAMATANI et al., 2013) and *Arabidopsis thaliana* mutants *sgr* (REN et al., 2007), *nyc1* and *nol* (HORIE et al., 2009), *pph* (SCHELBERT et al., 2009), *nyc3* (MORITA et al., 2009), *lls1* (PRUZINSKÁ et al., 2003), *acd1* (TANAKA et al., 2003) have been described.

The Chl retention can be caused by a defect in any of the Chl catabolic enzymes (CCEs) or due to the absence of the SGR protein. Alterations in the Chl degradation pathway often result in type C or type D stay-green phenotypes. In rice, absence of either *NYC1* or *NOL* leads to the type C stay-green phenotype during natural senescence and under dark-induced senescence (KUSABA et al., 2007; SATO et al., 2009). In contrast, in Arabidopsis only the absence of *NYC1* results in the type C stay-green phenotype while the absence of *NOL* does not affect Chl b degradation (HORIE et al., 2009).

Arabidopsis and rice mutants defective in *PPH* also exhibit a stay-green phenotype (SCHELBERT et al., 2009; MORITA et al., 2009). The Chl breakdown in these mutants is slowed down by the accumulation of phein a that seems to suppress Chl breakdown activity in a feedback manner (SCHELBERT et al., 2009; MORITA et al., 2009). Although the Chl degradation is impaired in the *pph* mutants, photosynthetic activity during senescence decreases just as fast as in the wild type. Thus, the *pph* mutant can be classified as a type C stay-green (KUSABA et al., 2013).

The *hcar* Arabidopsis mutant also results in a stay-green phenotype (MEGURO et al., 2011) by the accumulation of a small amount of 7-hydroxymethyl-Chl and a large amount of pheide a during induced senescence. It is not clear why the *hcar* mutant accumulates pheide a, as well as 7-hydroxymethyl-chlorophyll. Therefore, the

accumulation of pheide a results in the generation of a high level of reactive oxygen species that leads to cell death which classifies this mutant as a type D stay-green (KUSABA et al., 2013).

As in the *hcar* mutant, stay-green plants that accumulate high levels of pheide a during dark-induced senescence present substantial cell death (TANAKA et al., 2003; PRUZINSKÁ et al., 2003). For example, rice *lls1* and Arabidopsis *acd1* mutants, as well as Arabidopsis transgenic plants with reduced PAO activity accumulate pheide a and can also be classified as type D stay-green.

As mentioned above, *sgr* mutants retain Chl during senescence, similar to mutants defective in CCEs. Originally, absence of SGR was considered to inhibit Chl breakdown, but recent data indicate that SGR is not directly involved in a Chl catabolic step; instead, it is required for the dismantling of photosynthetic Chl–protein complexes, thus allowing Chl-breakdown enzymes to access the Chl molecules, with the simultaneous proteolytic digestion of the apoproteins (HÖRTENSTEINER, 2009, 2013; SAKURABA et al., 2012). Thus this protein is likely a ‘‘master’’ regulator of Chl degradation (JIANG et al., 2007; PARK et al., 2007; REN et al., 2007; AUBRY et al., 2008; SAKURABA et al., 2012).

SGR is a senescence-associated gene that is typically up-regulated during senescence. The proteins encoded by *SGR* genes from different species are 25−30 kDa in size and lack a clear transmembrane domain or any other known protein domains other than the one defined by the SGR family (PF12638) (Ren et al., 2007). The SGR protein is reported to interact with light harvesting complexes (LHC) and Chl-degrading enzymes, including NYC1, NOL, HCAR, PPH, PAO, and RCC reductase (PARK et al., 2007; SAKURABA et al., 2012).

The mutants already described with defects in Chl breakdown, can mostly be classified as type C or D stay-green plants. Although degradation of components of the photosynthetic apparatus is delayed in *sgr* mutants during senescence, other proteins in the chloroplast are degraded as fast as in wild-type plants (PARK et al., 2007; SATO et al., 2007). Thus, *sgr* mutants, as well as the CCEs defective stay-green mutants are classified as type C stay-green plants (KUSABA et al., 2013).

Type A and type B stay-green phenotypes are observed in mutants that have defects in other metabolic or phytohormone signaling pathways. Some examples of functional stay-green mutants that could be classified in these two categories of stay

green plants are listed here: the Arabidopsis *ore1*, *ore4-1*, *ore7*, *ore9*, *ore12* and *dls1*(YOSHIDA et al., 2002; LIM et al., 2007) the *Oriza sativa* SNU-SG1 (YOO et al., 2007); the *Triticum aestivum* XN901 (GONG et al., 2005); the *Triticum durum* 139, 142, 196 and 504 (SPANO et al., 2003) and; the *Zea mays* FS854 (THOMAS; HOWARTH, 2000). The cause of Chl retention in some of these mutants is not yet determined, but in all cases they don't seem to be related to any of the CCEs or the SGR protein.

Most of the mutations causing the stay-green phenotype described so far mention Chl retention in leaves. However, the most famous *sgr* mutant, the Mendel's green cotyledon pea, one of the mutants used in determining the law of genetics, retains greenness of leaves during senescence, but also of the cotyledons during seed maturation (SATO et al., 2007). Interestingly, there are two other stay-greens that also retain Chl in seeds: the *d1d2* stay-green mutant in soybean (GUIAMÉT et al., 1991; CANFIELD et al., 1995) and some of the Arabidopsis *abi3* mutants (NAMBARA et al., 2000).

During seed maturation, ABI3 regulates several processes, such as dormancy acquisition, long-term storability, as well as loss of Chl. Although multiple *ABI3* alleles have been identified with various degrees of ABA insensitivity, not all of these alleles display the green seed phenotype exhibited by the severe alleles like *abi3-1* (CLERKX et al., 2003) and *abi3-6* (DELMAS et al., 2013).

In soybean, some stay-green mutants have been isolated. A cytoplasmic gene, *cytG*, or homozygosity for two recessive alleles, *d1* and *d2*, at two different nuclear loci, besides causing Chl retention in leaves during senescence, also cause pod walls, seed coats, and embryos to stay green at maturity (GUIAMÉT et al., 1991). Homozygosity at both nuclear loci is required because of their redundant function. The two are paralogous (duplicated) loci in the tetraploid soybean genome (FANG et al., 2014). The dominant nuclear gene *G* inhibits yellowing of the seed coat but not the leaves, pod walls, or embryos. Chl retention is associated with a delayed decline of photosynthesis in the genotype *GGd1d1d2d2*, but not in *d1d1d2d2* or *cytG* (GUIAMÉT et al., 1991).

Therefore, the analysis of the genes already described as causing stay-green phenotypes could be a successful starting point to understand the Chl retention in soybean seeds as will be shown further in this study.

3. Materials and Methods

3.1. Plant materials

Two cultivars of soybean were used to produce the seeds used in this study. The cultivars varied in susceptibility to Chl retention in the seeds. The cultivar MG/BR 46 (Conquista) (late cycle) was used as the susceptible material and the cultivar BRS 133 (semi-early cycle) was used as the tolerant genotype (PÁDUA; CARVALHO; et al., 2009).

3.2. Growth conditions

The plants (4 plants/pot) were grown in pots of 15l filled with sandy loam soil. The soil was fertilized and limed according to the crop requirements. The analysis of the phenological stages of the plants were based on the scale proposed by Ritchie et al. (1982) with slight modifications that will be discussed in the results section in order to better characterize the seed maturation (Table 1).

During the vegetative phase both cultivars were grown in a polytunnel, subjected to the local environmental conditions (Botucatu, SP – Brazil). During this phase the climate condition of this region could be considered as non-stressed with an average temperature of 24.7ºC (Figure 2 and Figure 3). And to completely characterize a non-stressed environment the plants had a free water supplement (field capacity). At R5.5 (Figure 4) part of the pots were maintained under these non-stressed conditions and part was transferred to a greenhouse (Van der Hoeven, Brazil) with temperature control for the

application of stress. The external environment exerts some influence on the temperature inside of the greenhouse, therefore the variation in this experiment was not significant. In the stressed conditions the average temperature varied from 25.7 to 39.5°C for the susceptible cultivar and from 24.4 to 38.7°C for the tolerant one (Figure 2 and Figure 3). The plants in stressed conditions were only watered when wilting was observed. At this moment plants were watered to bring the soil to field capacity.

3.3.Sampling points

The seeds were harvested at three stages of maturation (R6, R7 and R8) solely based on plant and pod characteristics (Table 1). At each stage the four plants in the pot (six pots) were cut and the pods were removed. A post-harvest selection was made in order to remove 'off-color' pods, based on the characteristic color of the pods in each stage of maturation (Figure 5) in an attempt to reduce variability of maturity among seeds in the same sample.

Combining the environmental conditions (non-stressed and stressed) and the harvesting points (R6-R7-R8), there were six different seed samples for each cultivar and six replicates. After the analysis of green seed production (% of green seeds) the six replicates of each sample were grouped two by two to constitute three replicates, simply to reduce the number of replicates for the following physiological and molecular analysis. The appearance of the pods and seeds constituting non-stressed samples in each of the three stages of maturation is displayed in Figure 5.

3.4. Percentage of green seeds

The percentage of green seeds produced by the susceptible and tolerant cultivars was visually analyzed considering the whole seed production (4 plants/pot x 6 pots), thus the analysis was done in six replicates. In the R6 sample (stressed and non-stressed), seeds were considered as 'green' if there was no sign of Chl degradation and they were still completely green. At R7, when the Chl degradation had already started and the samples are characterized by a mixture of greenish and light-yellow seeds, the greenish ones were considered as 'green'. And at R8 when the seeds must be yellow, any sign of green pigmentation classified seeds as 'green'.

Table 1. Description of the vegetative and reproductive stages of soybean (RITCHIE et al., 1982).

Stage of Maturation	**Nomination**	**Description**
VE	Emergence	Cotyledons above the soil surface
VC	Cotyledon	Cotyledons fully unfolded
V1	First node	Unifoliate leaf fully unfolded
V2	Second node	First trifoliate leaf fully unfolded
V3	Third node	Second trifoliate leaf fully unfolded
Vn	nth node	Ante-nth trifoliate leaf fully unfolded
R1	Beginning bloom	One open flower at any node on the main stem
R2	Full bloom	Open flower at one of the two uppermost nodes on the main stem with a fully developed leaf
R3	Beginning pod	Pod is 5mm long at one of the four uppermost nodes on the main stem with a fully developed leaf
R4	Full pod	Pod is 2cm long at one of the four uppermost nodes on the main stem with a fully developed leaf
R5	Beginning seed	Seed is 3 mm long in the pod at the for uppermost nodes of the main stem with a fully developed leaf
R5.1	~10% filling	Seed is ~5mm long in the pod (perceptible to the touch)
R5.2	~11-25% filling	Seed is ~7mm long in the pod
R5.3	~26-50% filling	Seed is ~8mm long in the pod
R5.4	~51-75% filling	Seed is ~10mm long in the pod
R5.5	~76-99% filling	Seed is ~11mm long in the pod
R6	Full seed	Pod containing a green seed that fills the pod cavity at one of the four uppermost nodes on the main stem with a fully developed leaf (plant with green leaves)
R7	Begin of maturation	One normal pod on the main stem that has reached its mature pod color
R8	Full maturity	Ninety-five percent of the pods have reached their mature pod color

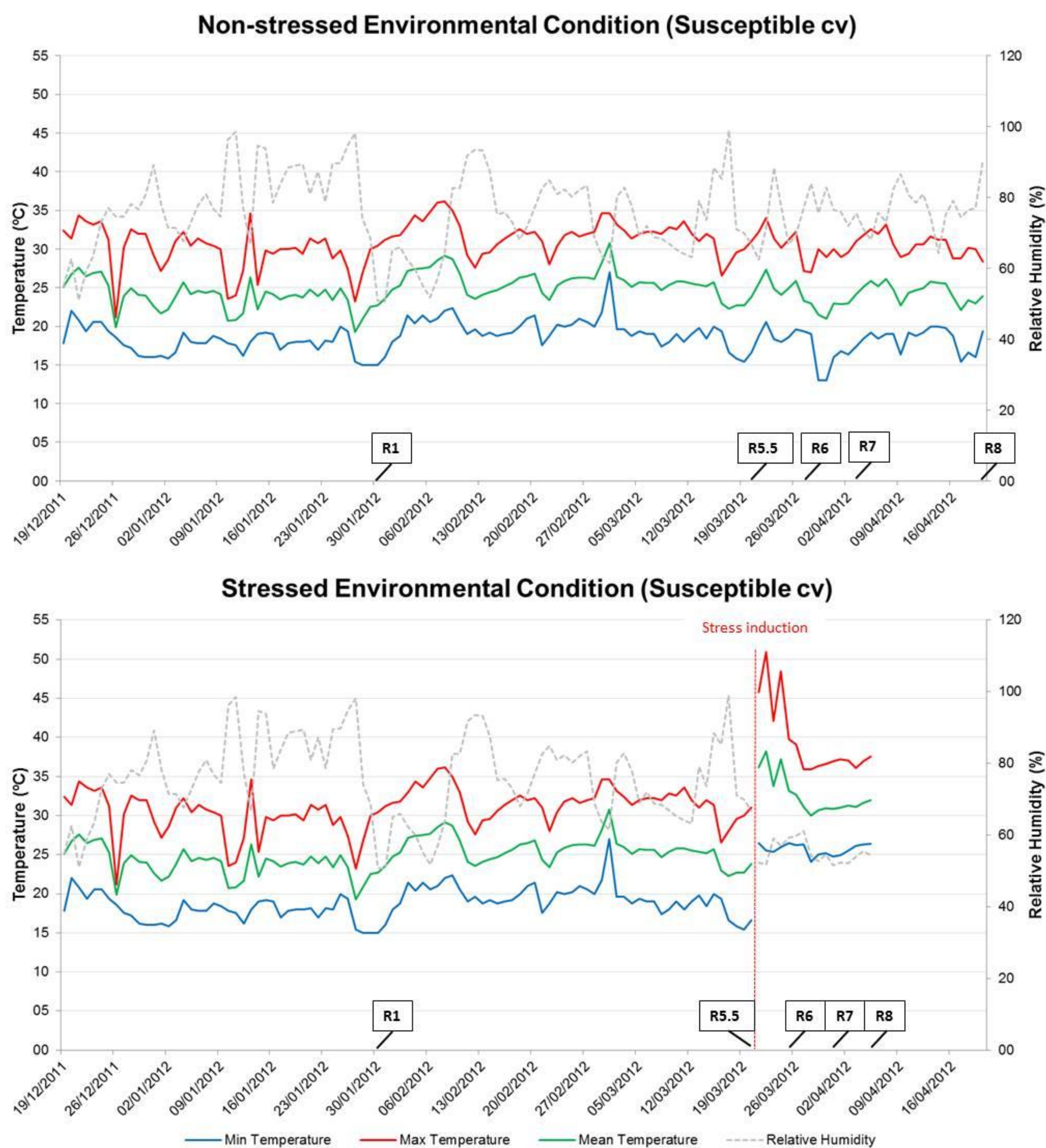


Figure 2. Average temperatures and relative humidity during the life cycle of the susceptible cultivar under non-stressed and stressed conditions.

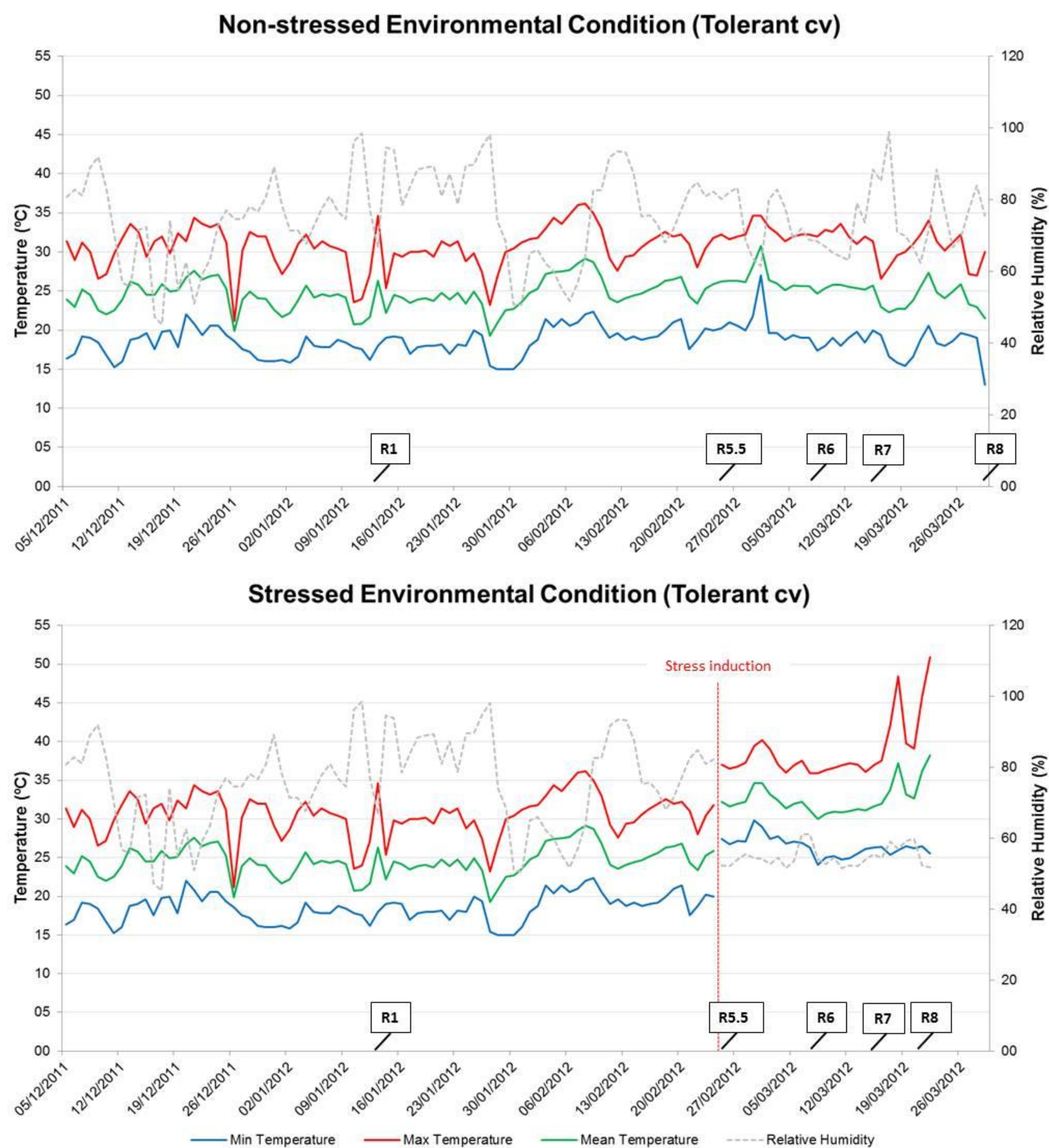


Figure 3. Average temperatures and relative humidity during the life cycle of the tolerant cultivar under non-stressed and stressed conditions.

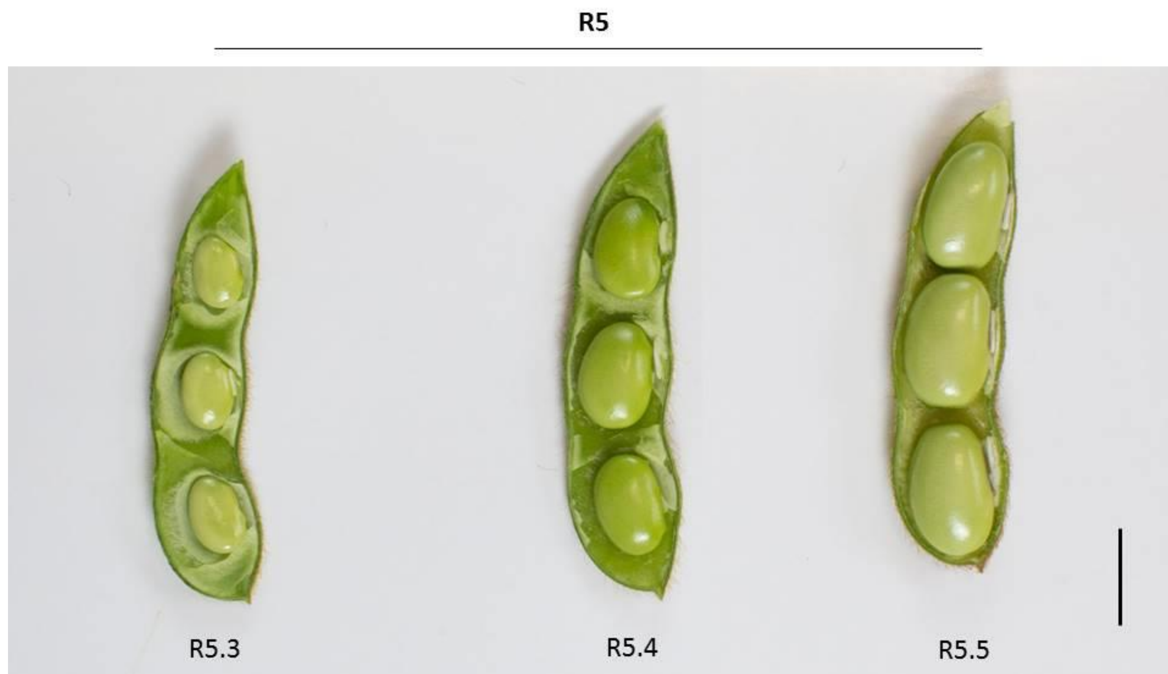


Figure 4. Reproductive stage R5 and the progression of seed development in R5 (R5.3; R5.4; R5.5). Black bar represents 1cm.

3.5. Chlorophyll quantification

Materials – Samples of non-stressed and stressed seeds of the susceptible and tolerant cultivars harvested at the three stages of maturation were lyophilized and ground to a powder for the quantification of Chl a and b by high performance liquid chromatography (HPLC). Both Chl a and b standards were purchased from Sigma (USA). All the solvents used were HPLC grade.

Extraction of Chls – Extraction of Chls was performed under green light according to Teng and Chen (1999) with adaptations. Ground seed samples of 0.5 to 2 g were placed in a Falcon tube (50ml), to which 5ml of methanol was added. The mixture was homogenized with a homogenizer for 30 s and centrifuged at 3000 rpm (4ºC) for 3 min. The supernatant was collected in another Falcon tube. In a second step the same procedure was repeated with acetone, and this was repeated several times until the extract became colorless. All the methanol/acetone extracts were pooled and brought to a volume of 25 or 50 ml with the addition of methanol and the solution was filtered through a 0.2 µm membrane filter for HPLC and placed in an amber vial for immediate subsequent injection. The sample weight and methanol/acetone volume depended on the pigmentation intensity. The extraction of Chls maintained the ratio of 1:100 (w/v) for R6 samples (intense green),

1:50 (w/v) for R7 samples (light green) and 1:12.5 (w/v) for R8 samples depending on the intensity of the pigment color (Chl concentration).

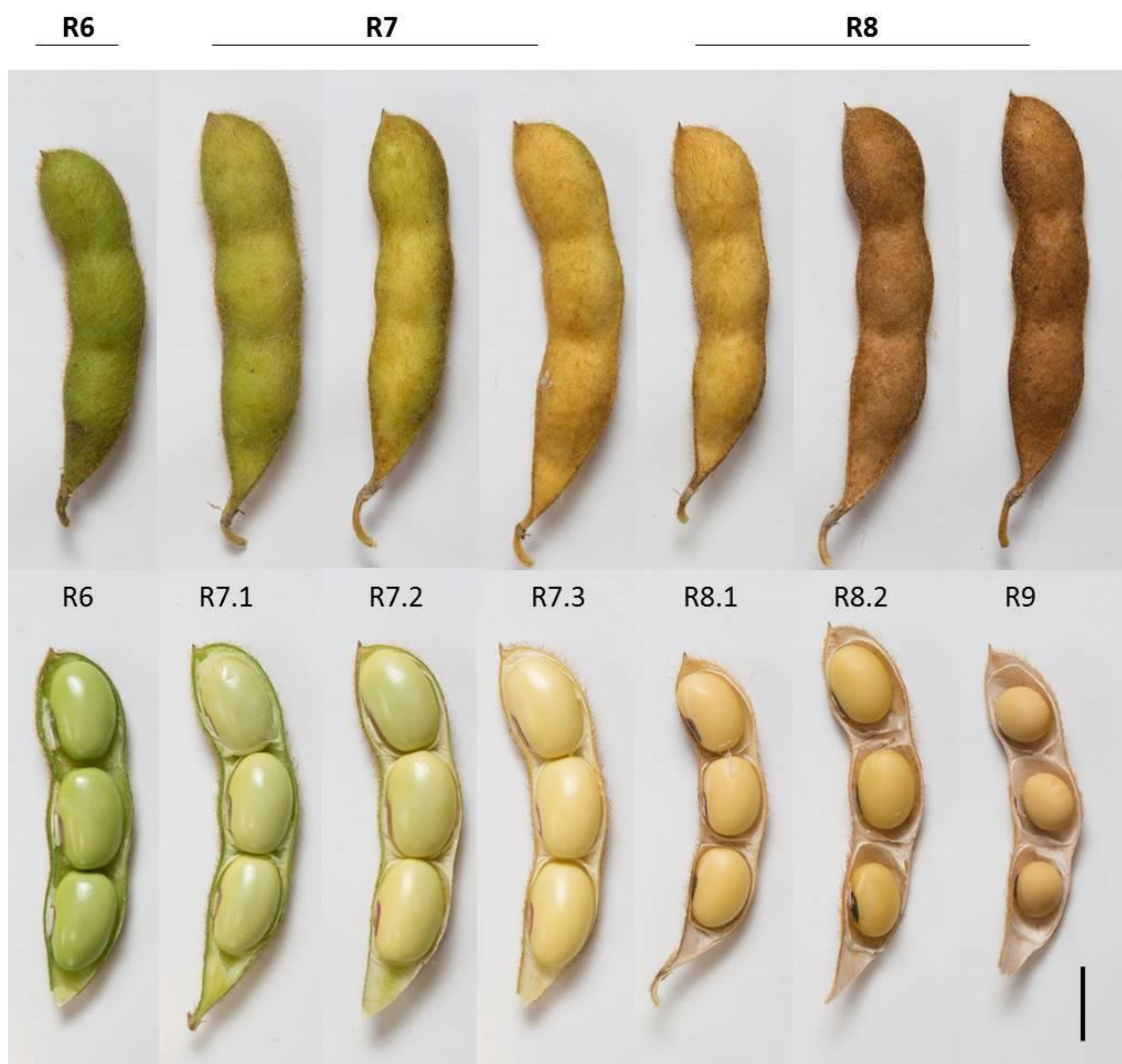


Figure 5. Pod and seed characteristics at the reproductive stages R6, R7 and R8. Subdivisions of R7 and R8 considering only seeds morphological characteristics are proposed and discussed in the results section. Black bar represents 1cm.

Analysis of Chl by HPLC – Analytical separations were performed on a Shimadzu Shim-Pack VP-ODS column (150 x 2.0 mm - serial number 6102708). Pigments were eluted using a linear gradient in 15 min, with a two-solvent system: (A)

methanol:ammonium acetate (80:20 v/v) and (B) methanol:acetone (80:20 v/v). The injection volume was 25 µl. The fluorescence detector (Shimadzu RF-10XL) was operated at an excitation of 440 nm, an emission of 660 nm and a flow rate of 0.8 ml/min (ALMELA et al., 2000).

The calibration curves were made from 0.01 to 5.0 mg/l for both Chls. High linearity was obtained with correlation coefficients of 0.99. Chl a and b were then quantified using the respective calibration curves.

3.6. Seed quality assessment

To assess the physiological quality of the seeds, viability and vigor of non-stressed and stressed seeds of both cultivars in the three stages of maturation were analyzed as described:

Germination – was evaluated in four replicates of 50 seeds each, in paper rolls at 25ºC. The germination percentage was scored by counting radicle protrusion (1mm).

Normal and abnormal seedlings – normal and abnormal seedling formation was scored after eight days of imbibition, using seedlings characteristics parameters as described in Brasil (2009).

Dormant seeds – Seeds were considered dormant if they didn't imbibe, or imbibed without germination after eight days of imbibition. Additional testing of viability was done by the tetrazolium test.

T50 – the time required for 50% of viable seeds to germinate was analyzed by daily counts of radicle protrusion. The calculation was performed by analyzing cumulative germination data using the curve-fitting module of the 'Germinator software package' (JOOSEN et al., 2010).

Seedling, root and shoot length – the length of the roots and shoots of normal seedlings was measured after eight days of imbibition. Four replicates of ten seedlings were used in this test. The average length of seedling, root and shoot was calculated for each replicate.

Seedling dry weight – seedlings were placed to dry in an oven with air circulation at 80°C for 24 hours and the average weight of each replicate was calculated.

3.7. RNA extraction

Total RNA extraction was performed according to the NucleoSpin® RNA Plant kit (Macherey-Nagel) protocol in three replicates, using non-stressed and stressed seeds of the susceptible and tolerant cultivar at three stages of maturation (R6-R7-R8) with at least 15 seeds of each biological sample (replicate). RNA integrity was assessed by analysis on a 1% agarose gel, and RNA sample quality and concentration were additionally assessed using a Nanodrop ND-1000 (Thermo Scientific).

3.8. Microarray analysis

For the microarray analysis, three biological replicates of total RNA from seed samples of the susceptible cultivar (MG/BR 46) were used. After cDNA synthesis, done according to Affymetrix protocol (Affymetrix®, GeneChip® 3' IVT Express Kit user manual) the samples were hybridized to Affymetrix microarrays (Soybean Genome GeneChip Array). The hybridization process was done by Molecular Core Co. (Unifesp – SP). In total 18 arrays were used (3 stages of maturation X 2 environmental conditions X 3 replicates).

Quality control and pre-processing analysis of the microarray results was done using a tool of the open source package ArrayAnalysis.org that is freely available from http://www.arrayanalysis.org (EIJSSEN et al., 2013). RMA-normalized data was filtered to remove probesets with a signal below a minimum cutoff (expression value < 3) in at least one of the conditions analyzed.

Probesets were then assigned to dominant patterns (DPs) using the FANNY method in R (http://cran.r-project.org/web/packages/cluster/cluster.pdf) (BELMONTE et al., 2013) based on a minimum Pearson correlation of 0.85. The number of dominant patterns was determined by the analysis of K choices from 1 to 30. The final set of patterns was analyzed and 9 DPs were selected that were unique or not overlapping other DPs.

Probesets assigned to each DP were used to identify enriched gene ontology (GO) terms. The over-representation analysis (ORA) was done using agriGO (GO Analysis Toolkit and Database for Agricultural Community) (DU et al., 2010). For the ORA, the p-values were adjusted for multiple testing with a significance level of 0.01.

To get a shorter list of candidate genes related to stress and thus Chl retention in soybean seeds, two comparisons were carried out separately using R. For both comparisons data was corrected for multiple testing error (FDR). A 2-fold change and significant level of 0.05 (p-value≤0.05) were used for filtering.

Probesets in the final list of both comparisons were addressed to GOs and an ORA was performed as described above. In order to visualize the overall differentially expressed genes (p-value≤0.05), the Page-Man/MapMan package (http://MapMan.gabipd.org) was used (THIMM et al., 2004), with visualization by the seed specific molecular network pathway (JOOSEN et al., 2011).

The best candidate genes were chosen based on important ontologies for the biological question (stress and Chl presence). These genes were then validated by RT-qPCR analysis in stressed and non-stressed seeds of the susceptible and tolerant cultivar in the three stages of maturation.

3.9. Quantitative PCR (RT-qPCR) conditions

The RNA used for gene expression analysis by RT-qPCR was extracted from seed samples that went through another (more stringent) classification of stages of maturation, based on seed color, in order to provide greater accuracy. cDNA was then synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacture's protocol. iQ-SYBRGreen-Supermix (Bio-Rad) was used for gene expression analysis on an MyIQ RT-qPCR machine (Bio-Rad) or LuminoCt SYBR Green qPCR ReadyMix (Sigma) for the analysis on an Eco Real-Time PCR system (Illumina).

The RT-qPCR program for both machines consisted of a first step at 95°C for 3 min, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. The primers used (Table 2) were preferably designed in the 3' end of the transcript and, if possible, spanning an intron–exon border. The Tm of the primers was between 58 and 62ºC except a few primers with slightly lower Tm. Routinely a melting curve analysis was performed after the qPCR run (between 55 and 95ºC with 0.5ºC increments each 10 s). For all primers, a single peak was observed, confirming the synthesis of a single product.

Primer efficiency was calculated performing a 10X dilution series and the slope of the standard curve was translated into an efficiency value. All primers

showed efficiencies between 90 and 110%. Ct values obtained from iQ5 software (Bio-Rad) were analyzed with qBase+ (Biogazelle, Zwijnaarde, Belgium). Ct values obtained from the Eco Real-Time software (Illumina) were analyzed in an excel sheet by the ΔΔCt method. RT-qPCR data of each gene of interest were normalized against the two most stable reference genes, chosen as described below.

3.10. Reference gene identification

As the "classical" reference genes for soybean found in the literature were not stable for gene expression analysis of our samples (data not shown), the identification of new reference genes was done based on microarray data. The most stable transcripts were selected based on the datasets from three microarray experiments: this study, Hudson (2010) and Asakura et al. (2012) (the two last ones are available at http://www.ncbi.nlm.nih.gov/geo/ as GEO Series ID GSE18827 and GSE26443, respectively). These datasets were chosen for being performed with developing soybean seeds produced in different environmental conditions, which would improve the power of the analysis and the quality/stability of the identified reference genes.

For the mining of stably expressed genes, gcRMA normalized data of in total 50 microarrays were used. Over the entire set of arrays the average expression and standard deviation (SD) were calculated per gene. For each gene, the coefficient of variation (CV) was calculated by dividing the SD by the mean expression. Genes with low CV values are more stably expressed compared with genes with high CV values. The gene set was filtered for an average expression between 7 and 11, what would coincide with the range of expression of the target genes chosen in this study. Six genes with the lowest CV values were tested (Table 3). Their expression stability was calculated by geNORM (VANDESOMPELE et al., 2002) and the two most stable genes (Prot and 60S) were used for RT-qPCR expression data normalization in this study. Genes with a high M value are less stably expressed compared with genes with a low M value.

Table 2. Primers sequences used for the target gene study.

Gene		Forward	Reverse	Reference
NYC1_1	Glyma07g09430	TCGGGAGTTTCTTCTTTCTGGA	ACAAACATCACAAGCAATGCCTACA	Fang et al. (2014)
NYC1_2	Glyma 09g32370	GCAACAACAAGCCATCTCAAA	GGCCATGGATGTGATGATAGAA	This study
PAO_1	Glyma 11g19800	CTTGTGTTAAGATTCCTCAGGCTT	ACCATTCTCATCAGGCCATACA	Fang et al. (2014)
PAO_2	Glyma 12g08740	CAACCATTGCCATCAACTGTT	TTGCACAAAACACAACTGTTGTA	Fang et al. (2014)
RCCR_1	Glyma 14g01620	GACCCACAACAACCAA	TGCAAGGAAGAAGCTGAGT	This study
RCCR_2	Glyma02g47120	CTCTCTGTTCCATCTTCTTCTTTG	GAAACGAGATCAACCATGAGG	Fang et al. (2014)
PPH_1	Glyma09g36010	TGGTGGAGTTTATTATACCAA	CCAATCACATAGCCATCGC	Fang et al. (2014)
PPH_2	Glyma11g16070	GCTGCCAAGTTGTGAACTCA	AATCTCACAGGCGCACATGC	Fang et al. (2014)
PPH_3	Glyma12g01320	ATGCTGTCAAGCAGTGGATAT	TCTCTTTGCAGCTCCTTATTG	Fang et al. (2014)
D1	Glyma01g42390	CAAGGAAGAGAGTGAGCAAGA	CACTGAATTGGGCTTAACGTC	Fang et al. (2014)
D2	Glyma11g02980	CACTCCTAAGAACAAAACCTTCAGT	TTTTCCCTACTGTGGAAGACGG	Fang et al. (2014)
CHL	Glyma10g00570	ACACCTCTTGTCTACGTCC	ACTCCTCCAACAAACCTTCTC	This study
LHCA	Glyma16g26130	ACCCATGGCACAACAACA	ACAGCACAGCGATACCAAC	This study
Cyt B6F	Glyma12g32580	CGTCCCTCTGTTGTCATGT	GGAGAGGTGATGGTGAAAAGTT	This study
psaA	---	AGCAACTCCCTTTTTCACC	GACCCGCTATCAAGAAAAGAAT	This study
psaB	---	TGGTGTTTATCAGTGGTGGT	TGATGATTGAGGCGGGATT	This study
psbA	---	GCAAACCTATAGCCGCAGA	GGATGGTTTGGTGTTTTGATGA	This study
psbB	---	CCCTCTGACCCTGTTCTT	ATATTCCAACCGCCCCAC	This study
psbC	---	CCTAGTAGTTTGCCGGAT	CACGTGGAAACGCTCTTTA	This study
psbD	---	AACGAAGTCATAGGCACG	CTTTGGGGTTGCTTTTTCC	This study

Table 3. Primers sequences used for the reference gene study.

Primer set	Annotation	Gene	Forward	Reverse	Frag. Length
UBQ	Ubiquitin	Glyma06g20310	CGAAGAGTGAGGGAAATTGT	GCCATCACTGCAACCAAA	102
SMP	Seed Maturation Protein	Glyma20g37670	ACAAGTCCTTCACCCCAAT	TAATCCTCCAAGCCCCACC	196
Aqua	Aquaporin	Glyma14g06680	TTTGTTGCCCTCTTCCCCC	CCCCACTTCTCACTCTCACC	114
Asp Aminoprot	Aspartate Aminotransferase	Glyma06g08670	TCTCCAACCTCCGCTTCT	GTTCAACTCGCCTCACTACA	184
Prot	Proteasome (20S subunit beta)	Glyma06g08260	CACCAACACACGATACAACT	TCCCAACCACCAACAATTAACC	122
60S	60S	Glyma15g42620	AGGCAGAGAAGGAGGAGAA	AAAGAAAACCTAGCACCCAAG	133

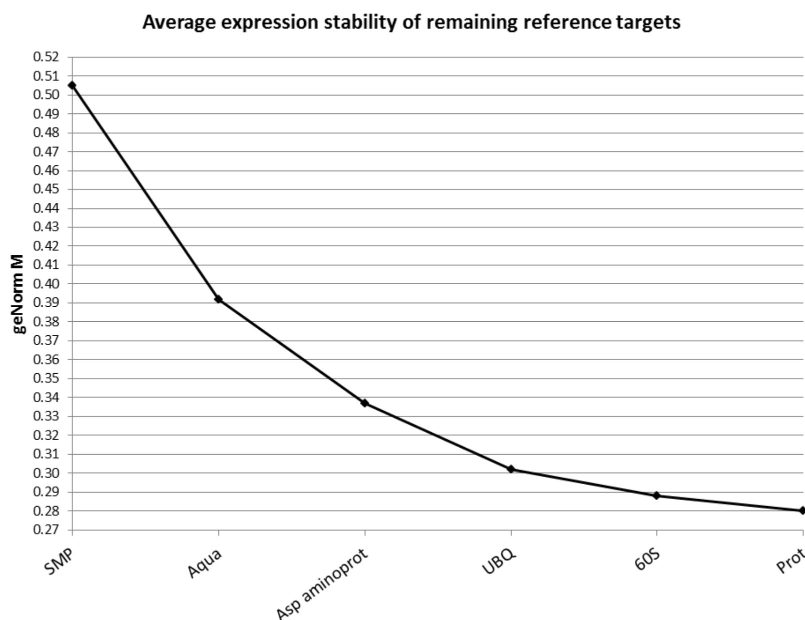


Figure 6. Expression stability of 6 reference genes for soybean seed research based on microarray expression data analyzed by geNORM.

3.11. Stay-green gene validation

In addition to the candidate genes found by microarray analysis, so-called stay-green genes (SGR) were also validated in stressed and non-stressed seeds of both cultivars in the three stages of maturation. These genes are important for the Chl degradation process and consequently closely related to the Chl retention phenomena as stated in the study of Chao-Fang et al. (2014). The list of SGR genes, including other target genes validated in this study can be found in Table 2.

3.12. Statistical analysis

An ANOVA test was performed on seed quality and gene expression data at the significance level of 5% ($p \leq 0.05$). A factorial 2 X 2 set-up (2 cultivars X 2 environmental conditions) was used and the analysis was done for each stage of maturation individually. The averages were compared by T test at 5% probability level.

4. Results and Discussion

4.1. Morphological aspects of soybean maturation under non-stressed and stressed conditions

During maturation under normal environmental conditions soybean seeds lose their green color by gradual Chl degradation (Figure 7). At the same time there is loss of water resulting in the reduction of size. In this study the maturation was evaluated in three stages (R6-R7-R8). For further studies we propose the subdivision of R7 and R8 based on visual analysis of the seed, which is possible due to clear differences in morphology. The subdivided stages (non-stressed seeds) are displayed in Figures 5 and 7.

At R6 the seeds are totally green. From that point onward Chl degradation is initiated, thus at R7.1 the degreening has already started, mainly in the embryonic axis and seed coat. At R7.2 there is an intensification of Chl degradation in the cotyledons where a mixture of green and yellow color is observed. At R7.3 the Chl has been completed degraded. From R7.3 towards the end of the maturation the major visible morphological change is the size reduction mainly caused by reduction of water content which will be discussed later. From R8.1 to R8.2 there is a darkening of the yellow color. At R9 the seeds have completed the maturation-drying and are at the point of harvesting maturity.

The embryonic axis is the first to degrade Chl during maturation (Figure 8) and is followed by the inner part of the cotyledons. Thus when the outside of a seed in R7.1 is still green, the degreening has already started from the inside.

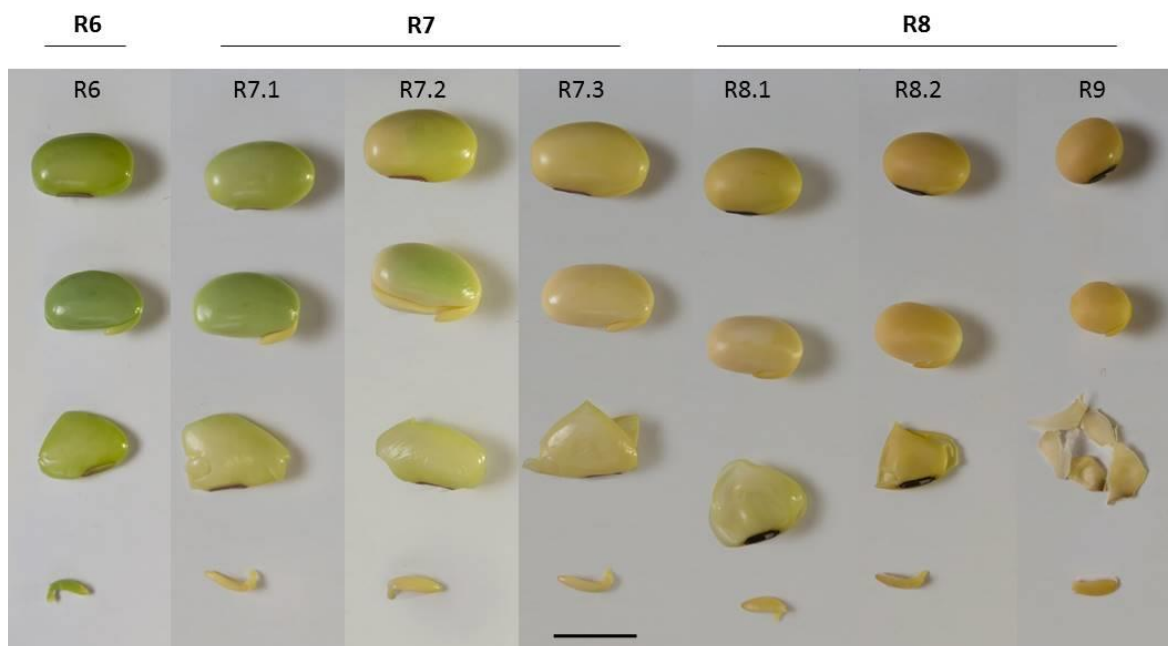


Figure 7. Soybean seed degreening during maturation under non-stressed environmental condition. From top to bottom: whole seed, embryo, seed coat and embryonic axis. Black bar at the bottom represents 0.5cm.

Figure 8. Internal aspect of soybean seeds during maturation under non-stressed environmental condition. Longitudinal section is displayed at the top; cross section is displayed at the bottom. Black bar represents 0.5cm.

In soybean plants under heat and drought stress leaf senescence starts earlier than in non-stressed environmental conditions. On the other hand the Chl degradation of the seed is retarded. This indicates that the stress is likely causing premature death of the plants, interfering in the speed of seed maturation, at least in respect to Chl

degradation. The analysis of the dates on which each of the cultivars, in the two environmental conditions, reached each phenological stage supports these differences in life cycle, viz. senescence of the plants (Table 4).

Table 4. Dates on which some of the phenological stages occurred for stressed and non-stressed plants of the susceptible and tolerant cultivar.

Phenological Stages	Susceptible		Tolerant	
	Non-stressed	**Stressed**	**Non-stressed**	**Stressed**
Sowing	19/12/2011	19/12/2011	05/12/2011	05/12/2011
R1	30/01/2012	30/01/2012	13/01/2012	13/01/2012
R5.5 (beginning stress)	21/03/2012	21/03/2012	25/02/2012	25/02/2012
R6	29/03/2012	26/03/2012	08/03/2012	08/03/2012
R7	03/04/2012	30/03/2012	15/03/2012	15/03/2012
R8	20/04/2012	5/04/2012	29/03/2012	22/03/2012
Cycle	122 days	107 days	114 days	107 days

The vegetative phase of the tolerant cultivar was 39 days long while that of the susceptible cultivar was 42 days long. The susceptible cultivar took 51 days from the beginning of the reproductive phase until the moment when the stresses were applied (from R5.5 onwards) while the tolerant cultivar took 43 days. As both cultivars were in the same environmental condition until R5.5, the stressed treatment was only characterized after that and the changes in days to reach the stage R5.5 is due to the variation in life cycle between the two cultivars.

After R5.5 the stresses had a strong effect on the time for maturation/senescence of the plants as the period from R5.5 to R8 was reduced 14 and 7 days for the susceptible and tolerant cultivars, respectively. In Arabidopsis *grs* mutants (green-seeded) grown under normal environmental conditions, the senescence of the fruits occurs normally while the speed of seed maturation is reduced within a normally maturing silique (CLERKX et al., 2003). Therefore, in this study the stress accelerated plants and pods senescence while seed degreening did not follow the same progress.

Based on this reduction in life cycle we can suggest that the high temperature and drought stress have, indeed, more effect on the susceptible material as it will also be shown by other characteristics than only speed of maturation/senescence. However, seed development and maturation involves much more than just morphological changes, degreening or moisture reduction. Hence, in future studies, other aspects of seed maturation, rather than only degreening, should be also analyzed for soybean seeds under stressful conditions, at the molecular and biochemical levels.

4.2. Chlorophyll retention in soybean seeds produced under heat and drought stress

The green seed production of soybean plants of the susceptible and tolerant cultivars grown under heat and drought stress was evaluated (Figure 9A). The Chl retention is mainly observed and accounted at the end of the maturation (R8). However to track the Chl retention we analyzed green seed percentage from R6 onward. At R6 100% of seeds produced by the tolerant cultivar under non-stressed and stressed conditions were green, though seeds of the susceptible cultivar had already started some Chl degradation, especially the stressed ones. At R7, both cultivars, under stress condition, had a smaller rate of seed degreening which accounted for higher levels of green seeds. At R8, when the Chl retention is a problem for seed quality, any greenish color characterized the seed as green. At this stage, the susceptible cultivar produced under stress a significantly higher level of green seeds, reaching 22% of green seeds against 1% produced by the tolerant cultivar. Pádua et al. (2009) reported production of green soybean seeds varying from 9% to 86%, depending on the severity of the imposed stress.

The significant difference in green seed production between stressed plants of the susceptible and tolerant cultivar confirms that the susceptibility of the soybean seeds to Chl retention is dependent on the genotype as was already shown by other researchers in canola and soybean (WARD et al., 1992; MAILER et al., 2003; PÁDUA; CARVALHO; et al., 2009).

Figure 10 illustrates the aspect of the seed samples of the susceptible and tolerant cultivars produced under non-stressed and stressed conditions. Non-stressed seeds are perfectly yellow (A and E) with a slight variance on the color tone due to specific characteristics of the cultivars. At B and F, samples of stressed seeds of

both cultivars display some percentage of green seeds. Especially for the tolerant cultivar seed sample (F), the reduced size of the green seeds can be observed.

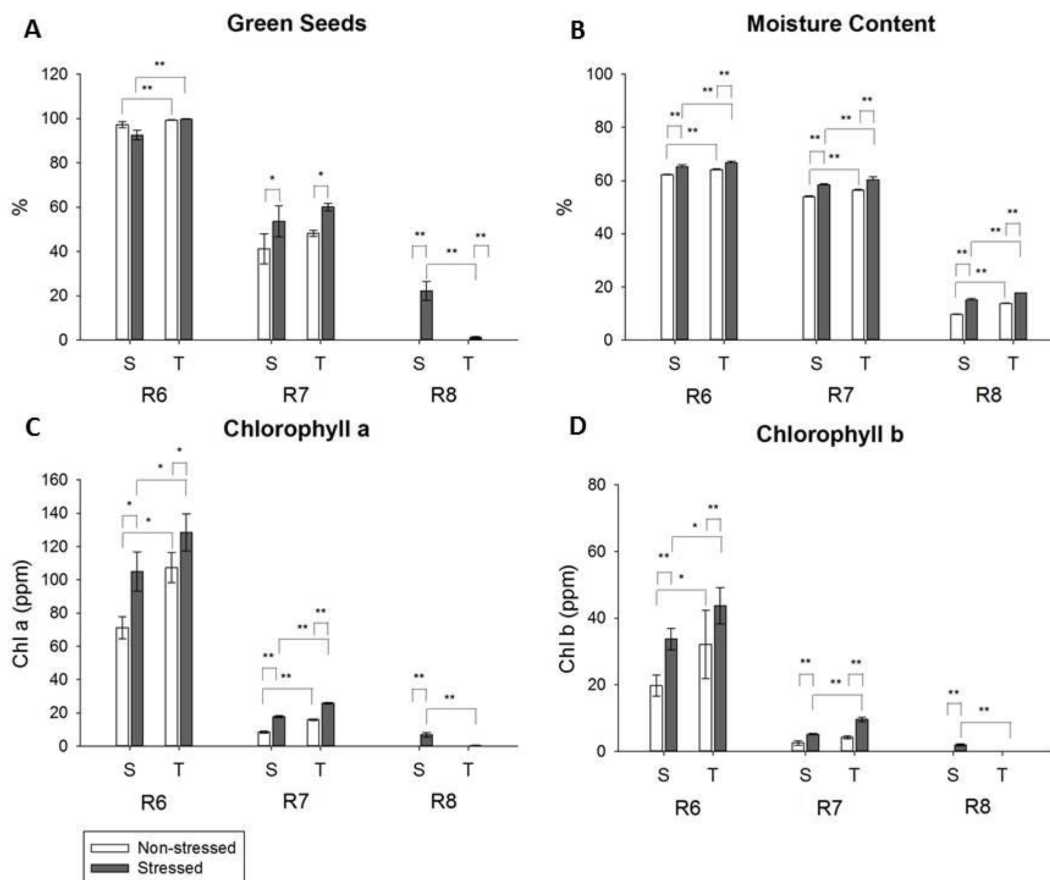


Figure 9. Percentage of green seeds (A), moisture content (B) and Chl a (C) and b (D) content of soybean seed samples produced under non-stressed (open bars) and stressed (closed bars) environmental condition by the susceptible (S) and tolerant (T) cultivar in three stages of maturation (R6-R7-R8). Bars represent the mean values ± SE of three independent biological replicates (six for the green seed percentage). Bars connected by brackets represent the statistical comparisons. Asterisks represents statistically significant differences (*, $P \leq 0.05$; **, $P \leq 0.01$).

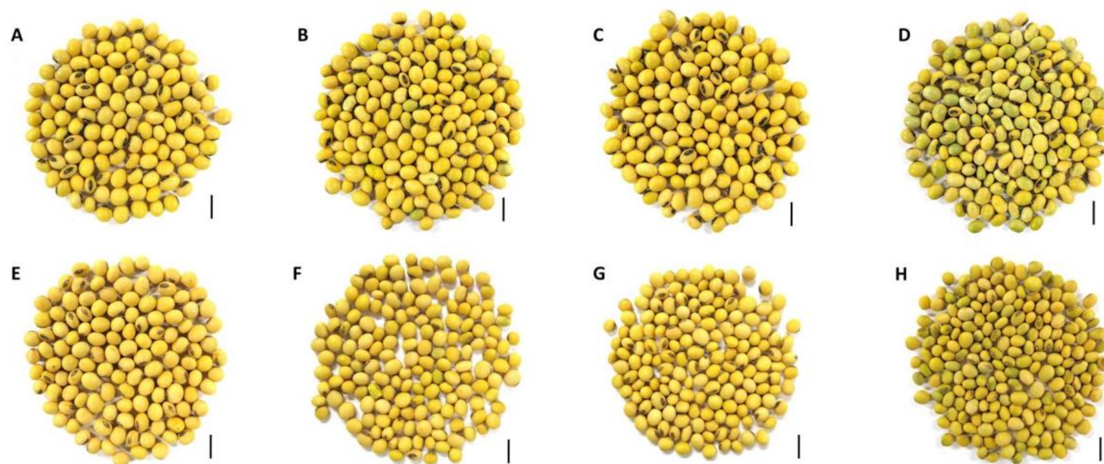


Figure 10. Non-stressed and stressed soybean seeds of the susceptible (top) and tolerant cultivars (bottom). A and E, non-stressed seeds; B and F, stressed seeds (original sample); C and G, yellow stressed seeds; D and H, green stressed seeds. Black bar on the right corner represents 1cm.

For C and G the portion of yellow (stressed) seeds was separated from the stressed seed samples B and F. The appearance does not clearly differ from the non-stressed seeds, except for the smaller size of the yellow stressed seeds of the tolerant cultivar (G) and some wrinkled seeds for the susceptible cultivar (C). The green seeds in D and H were also separated from the stressed seed samples B and F. Green seeds of the susceptible cultivar (D) are darker than the ones of the tolerant cultivar that tend to be greenish or not fully green. The size of the green seeds of the tolerant cultivar is smaller, which could indicate that these seeds were probably not fully developed/mature when they were harvested, although the plants were already dead and the pods yellow-brownish.

The characteristics of the green seeds of both cultivars can be better seen in Figure 11A. The Chl retention in stressed seeds of the susceptible cultivar is more severe, and differences in seed size can be observed clearly, strengthening the probable development/maturation disturbance in this seeds. Some of the green seeds are also wrinkled.

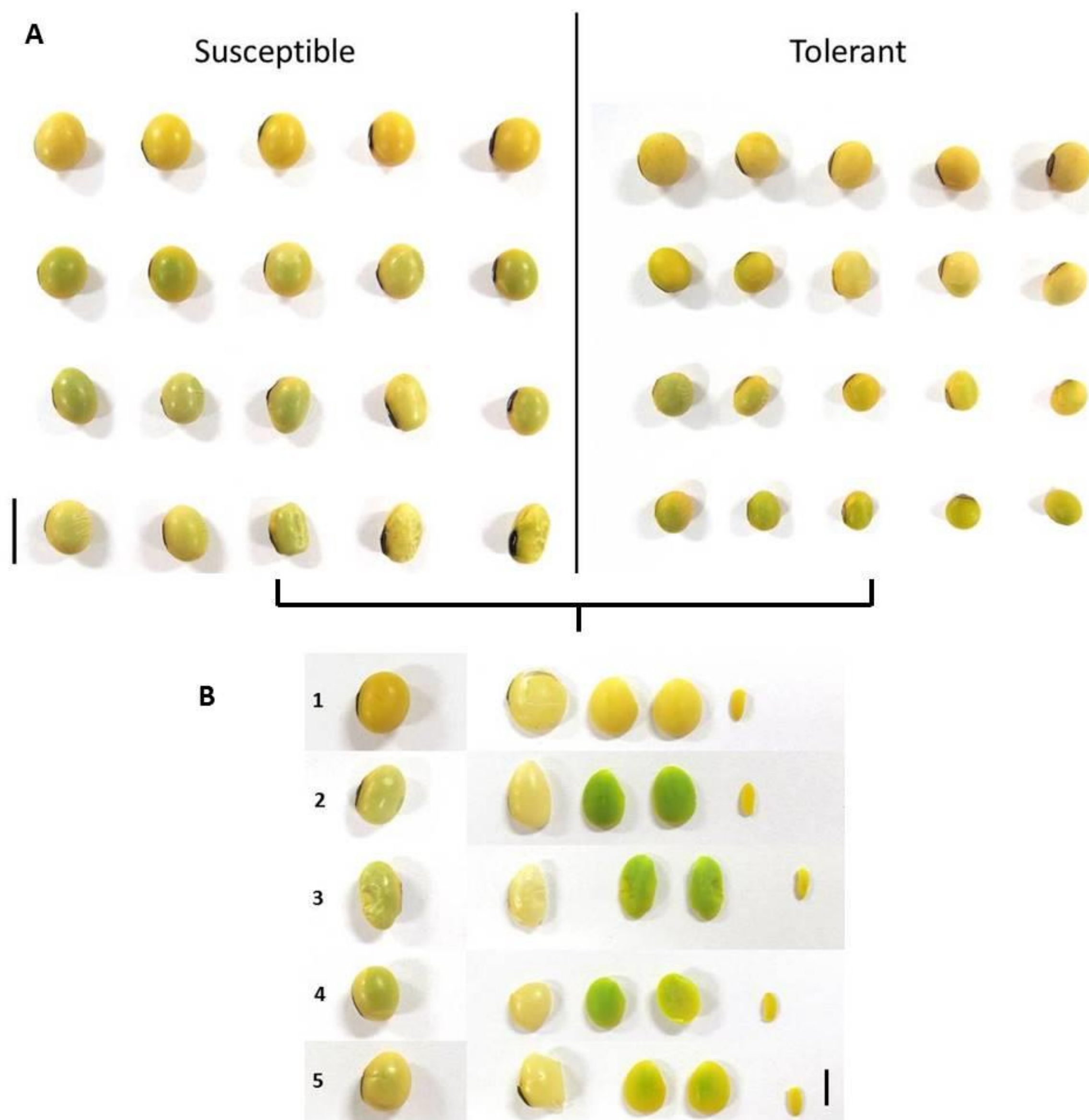


Figure 11. Characteristic of green soybean seeds. A: non-stressed (fist line) and stressed green seeds of the susceptible (left) and tolerant (right) cultivar. Black bar represents 1cm. B: Characteristics of different levels of Chl retention in individual soybean seeds of the susceptible cultivar. In each line from left to right: whole seed, seed coat, both cotyledons and embryonic axis. Line1: non-stressed yellow seed; lines 2-5: Chl retention in variable levels. Black bar represents 0.5cm.

Chl retention also varies within the different tissues of the seed. The main types of green seeds are shown in Figure 11B. At the top a seed without Chl (non-stressed) is shown (1). At line 2 an example of a seed that appears greenish but that

has almost completely green cotyledons and a yellow embryonic axis when the seed coat is removed. At line 3 an example of a greeninsh seed, similar to the one at line 2, though the cotyledons are wrinkled. At line 4, also an example of a greenish seed, but the green color of the cotyledons is slightly lighter. The seed at line 5 has only a small region of the cotyledon retaining Chl. Most analyzed green seeds didn't show retention of Chl in the seed coat, and the retention was never observed in the embryonic axis. As described before, under normal environmental conditions the Chl degradation starts from the embryo axis and seed coat. The Chl clearing then progresses through the cotyledons. This is probably the point at which the degradation of the pigment is mostly being interrupted.

For more accuracy in determining the Chl retention in stressed soybean seeds, Chl a and Chl b were measured by HPLC (Figure 9 C and D).

Chl a is always present at higher levels in the seeds than Chl b. At R6 and R7 both Chls were higher in stressed than non-stressed seeds of the susceptible and tolerant cultivars. Furthermore, in these stages the Chl content was higher in stressed and non-stressed seeds of the tolerant cultivar than stressed and non-stressed seeds of the susceptible one. At R8, there was only a difference between stressed and non-stressed seeds of the susceptible cultivar and the levels of Chl a and b in stressed seeds of this cultivar were significantly higher than in stressed seeds of the tolerant material. Once more, this indicates the susceptibility to Chl retention depending on the genetic material as discussed previously.

Chl a could even be detected in non-stressed seeds of both cultivars in R8 (

Table 5). At this stage of maturation, Chl b was only detected in stressed seeds of the susceptible cultivar. In maturing non-stressed soybean seeds the average ratio of Chl a/b is 3.5:1 for the susceptible cultivar and 3.57:1 for the tolerant cultivar. This ratio in the stressed seeds decreased to 3.3:1 for the susceptible and 2.82:1 for the tolerant cultivar.

In face of the expanding market, various classification systems have been used for international trade to control and standardize the production of soybean seeds for marketing. To evaluate soybean quality for the market, criteria such as damaged kernels, splits, foreign matter, moisture contents and the presence of green seeds have been used (SINNECKER et al., 2002). Routinely visual analysis of percentage of green seeds is used in order to judge the Chl retention. The content of green pigments has been estimated

by several physical methods for color measurement, which is indirectly related to Chl content (THOMPSON et al., 1996). High and significant linear correlations between color and total Chl were obtained over the whole soybean seed maturation period (SINNECKER et al., 2002). In the present study the percentage of green seeds (Figure 9A) was also coherent with the Chl content (Figure 9 C and D) showing that visual analysis of the percentage of green soybean seeds is a good indication of Chl retention.

Table 5. Total Chl, Chl a and b content and Chl a/b ratio in stressed and non-stressed seeds of two soybean cultivars (susceptible and tolerant) in three stages of maturation (R6-R7-R8).

Cultivars	Stages of Maturation	Environmental Condition	Chl A (mg/Kg)	Chl B (mg/kg)	Total (mg/kg)	A/B
Susceptible	R6	Non-Stressed	71,22±6.68	19,75±3.16	90,97±8.98	3.61
Susceptible	R6	Stressed	104,96±11.7	33,72±3.24	138,69±14.98	3.11
Susceptible	R7	Non-Stressed	8,48±0.61	2,50±0.70	10,98±0.19	3.39
Susceptible	R7	Stressed	17,69±0.60	5,16±0.25	22,85±0.62	3.43
Susceptible	R8	Non-Stressed	0,12±0.01	0,00±0.00	0,12±0.01	---
Susceptible	R8	Stressed	6,79±1.39	2,01±0.33	8,80±1.70	3.37
Tolerant	R6	Non-Stressed	107,29±8.95	32,12±10.20	139,41±10.20	3.34
Tolerant	R6	Stressed	128,43±11.2	43,76±5.41	172,18±15.81	2.94
Tolerant	R7	Non-Stressed	15,94±0.44	4,19±0.43	20,14±0.85	3.80
Tolerant	R7	Stressed	25,87±0.45	9,58±0.67	35,45±1.12	2.70
Tolerant	R8	Non-Stressed	0,11±0.00	0,00±0.00	0,11±0.00	---
Tolerant	R8	Stressed	0,45±0.03	0,00±0.00	0,45±0.03	---

Normal seed degreening is thought to be the result of a controlled process of plastid degradation; with changes in chloroplast ultrastructure and degradation of Chl-protein complexes occurring as the seed moisture content decreases (78–55%) during the pre-desiccation stage (JOHNSON-FLANAGAN; THIAGARAJAH, 1990). In stressed and non-stressed soybean seeds of the susceptible and tolerant cultivar there is an expected decrease in water content during maturation, with a more drastic water loss from R7 to R8 (Figure 9B).

The moisture content of stressed seeds is higher than non-stressed seeds of both cultivars at all stages of maturation. Therefore, in both stressed and non-

stressed seeds of the tolerant cultivar, the water content is always slightly higher. Normal Chl clearing in embryos seems to be related with a slow moisture loss. Under stressful environmental conditions, a rapid loss of moisture from fruit tissues followed by increased loss of seed moisture is likely to result in a seed moisture content that can no longer support Chl degradation (GREEN et al., 1998; BONHAM-SMITH et al., 2006; CHUNG et al., 2006). The role of rapid water loss in halting degreening is supported by an observation of Green et al. (1998) that green canola seeds can be produced by rapid drying of seeds in vitro.

In the present study the seed stage of maturation was determined visually, by the association of plant-pod-seed characteristics and not by following days after pollination or any molecular marker that would safely state the maturation condition. Thus, the higher moisture of stressed seeds probably indicates that these seeds are in a slightly earlier stage of maturation than the non-stressed seeds. Further investigations need to be done in order to understand the speed of maturation of soybean seeds produced under stress in comparison with the speed of leaf senescence and pod degreening.

4.3. The reduced quality of soybean seed lots containing green seeds

Seed quality is a complex trait and comprises many different attributes describing the condition of a seed lot. These attributes include germination characteristics, dormancy, seed and seedling vigor, uniformity in seed size, normal embryo and seedling morphology, storability, absence of mechanical damage, as well as the ability to develop into a normal plant (DICKSON, 1980; HILHORST; TOOROP, 1997).

To understand the effect of heat and drought stress and Chl retention upon soybean seed quality, viability and vigor of stressed and non-stressed soybean seeds were accessed (Figure 12A-H). Germination was not affected by stress/Chl as it reached almost 100% for non-stressed and stressed seeds of the susceptible and tolerant cultivar in all stages of maturation tested in this study (Figure 12A). Although there was a slight difference between non-stressed and stressed seeds of the susceptible cultivar at R7, at R8 this difference was no longer significant. At R8 the germination of non-stressed and stressed seeds of the tolerant cultivar was higher than the respective seeds of the susceptible one.

An important agronomical trait is the establishment of the initial stand in the field, therefore the percentage of normal seedlings was analyzed. This trait was more successful in distinguishing stressed from non-stressed seeds and also the two cultivars regarding to its susceptibility to the effects of stress/Chl on seed quality. The percentage of normal seedlings originating from non-stressed seeds increased during maturation. The highest values reached by non-stressed seeds were at R7, followed by a decrease in R8 (Figure 12B).

The physiological maturity of soybean seeds is achieved when the seed reach maximum dry weight at R7 (MARCOS-FILHO, 1979; TEKRONY et al., 1980). Thus, at R6 seeds are still completing reserve accumulation. Although they are able to germinate at this stage, most of them failed to develop into normal seedlings as can be observed by the lower percentage of normal seedlings compared with the other stages of maturation (Figure 12B).

The effects of stress/Chl are clearer at R6 and R8, since fewer stressed seeds of both cultivars developed into normal seedlings than non-stressed seeds. However, in R8 we observed that the effect of stress/Chl is dependent on the genotype since stressed seeds of the tolerant cultivar produced significantly higher percentage of normal seedlings as compared with stressed seeds of the susceptible one. It is important to highlight that the samples of stressed seeds of the tolerant cultivar contain significantly less green seeds (Figure 9A) and also lower levels of Chl (Figure 9C and D).

Since germination was not affected by stress/Chl, the reduced normal seedling percentages at R6 and also at R7 and R8 (stressed condition) is due to the number of abnormal seedlings (Figure 12C). The percentage of abnormal seedlings was reduced during maturation, but was, on average, higher in stressed seeds. At R6 and R7, stressed and non-stressed seeds of the susceptible cultivar generated higher levels of abnormal seedlings than the respective samples of the tolerant cultivar. At R8, there is a clear cultivar dependent formation of abnormal seedlings. In this stage, stressed seeds of the susceptible cultivar produced a higher percentage of abnormal seedlings than non-stressed seeds of the same cultivar and stressed seeds of the tolerant cultivar. Another interesting characteristic was the induction of dormancy in seeds of only the susceptible cultivar caused by the combination of heat and drought stress (Figure 12D). Although the percentage of dormant (stressed) seeds of the susceptible cultivar was relatively low (3%),

it is important to mention this fact since dormancy was eliminated from the 'modern' cultivars through the breeding programs.

Seed vigor was expressed as the speed of germination (t50) and seedling growth (seedling, root and shoot length) (Figure 12E-H). In R6 the t50 was higher for stressed seeds of both cultivars and higher for seeds of the tolerant one compared to the susceptible (Figure 12E). In R8 the difference between stressed and non-stressed seeds was only significant for seeds of the tolerant cultivar, although both stressed and non-stressed seed samples of this cultivar germinated faster than the respective seed samples of the susceptible cultivar. Thus, independent of the environmental condition, seeds of the tolerant cultivar are more vigorous. The shortest time required for germination of 50% of the viable seeds was in R7, therefore the comparisons between environmental conditions or cultivars showed unclear results when compared with the other two stages of maturation.

Seedling growth was another good parameter to distinguish the effect of stressed versus non-stressed environments on seed quality. The seedling length increased for stressed and non-stressed seeds of both cultivars during maturation (Figure 12F). The length of seedlings derived from stressed seeds was always shorter which can mainly be attributed to shorter roots (Figure 12 G), since there was no significant difference between shoots of stressed and non-stressed seeds (Figure 12H).

Seed quality is largely established during seed development and maturation, as a result of, often complex, interactions between the genome and the environment (LIGTERINK et al., 2012). This mechanism is part of the normal adaptation of plants to a varying environment and is aimed at maximizing the probability of successful offspring (HUANG et al., 2010). Depending on maturation conditions, storage conditions/time and genotype, seeds gradually lose vigor and viability. The aging for soybean can be considered to start earlier than we would normally assume since the time between the physiological maturity (R7) and the harvesting maturity (R8) in the field can be interpreted as a storage condition for the seeds. This could have effects on seed quality (COSTA et al., 1995; GREEN et al., 1998), as seeds are subjected to all sources of climate adversities.

Seed quality is a complex trait that is the result of a large variety of developmental processes, controlled by several genes and, therefore, is complex in genetic studies (CLERKX et al., 2003; LIGTERINK et al., 2012). Hence further research is needed to better understand the genetic basis of differences in seed quality.

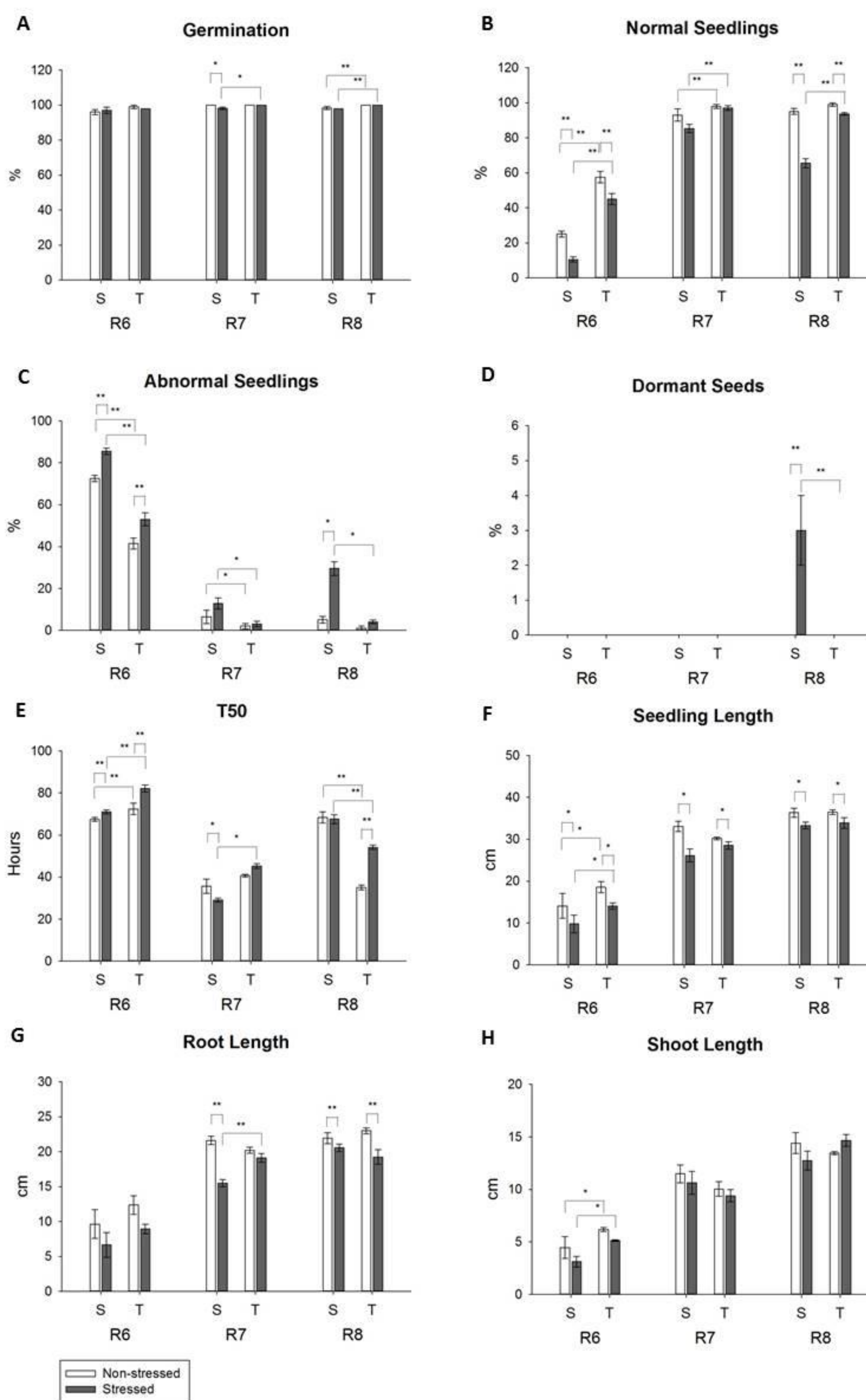


Figure 12. Germination and vigor parameters of non-stressed (open bars) and stressed seed samples (closed bars) of the susceptible (S) and tolerant (T) cultivars in

three stages of maturation (R6-R7-R8). A, germination; B, normal seedlings; C, abnormal seedlings; D, dormant seeds; E, t50; F, seedling length; G, root length; H, shoot length. Bars represent the mean values ± SE of three independent biological replicates. Bars connected by brackets represent the statistical comparisons. Asterisks represents statistically significant differences (*, $P \leq 0.05$; **, $P \leq 0.01$).

4.4. Global analysis of gene expression during soybean seed maturation under stressed and non-stressed conditions.

A global transcriptome analysis was carried out to identify changes in gene expression in soybean seeds of the susceptible cultivar, produced under heat and drought stress during the late stages of maturation. The microarray used in this study covers 37,500 soybean transcripts. After normalization and background removal data was filtered to remove probesets with a signal below a minimum cutoff (expression value < 3) in at least one of the conditions analyzed. From the total, 19,352 probesets remained for further analysis.

A principal component analysis (PCA) was performed which showed the global differences within stages of maturation and environmental conditions (Figure 13).

In the Y axis the gene sets were separated by environmental condition and in the X axis by stage of maturation. In R7, however, the separation in the Y axis (environmental condition) was not clear. This particular stage, considered as the physiological maturity, is a transition between two stages, R6 and R8 that are very different both morphologically and physiologically. R7, in this study, was a mixture of seeds with different degrees of Chl degradation (Figure 5.) which indicates seeds in slightly different maturation conditions as proposed previously by the subdivision of R7.

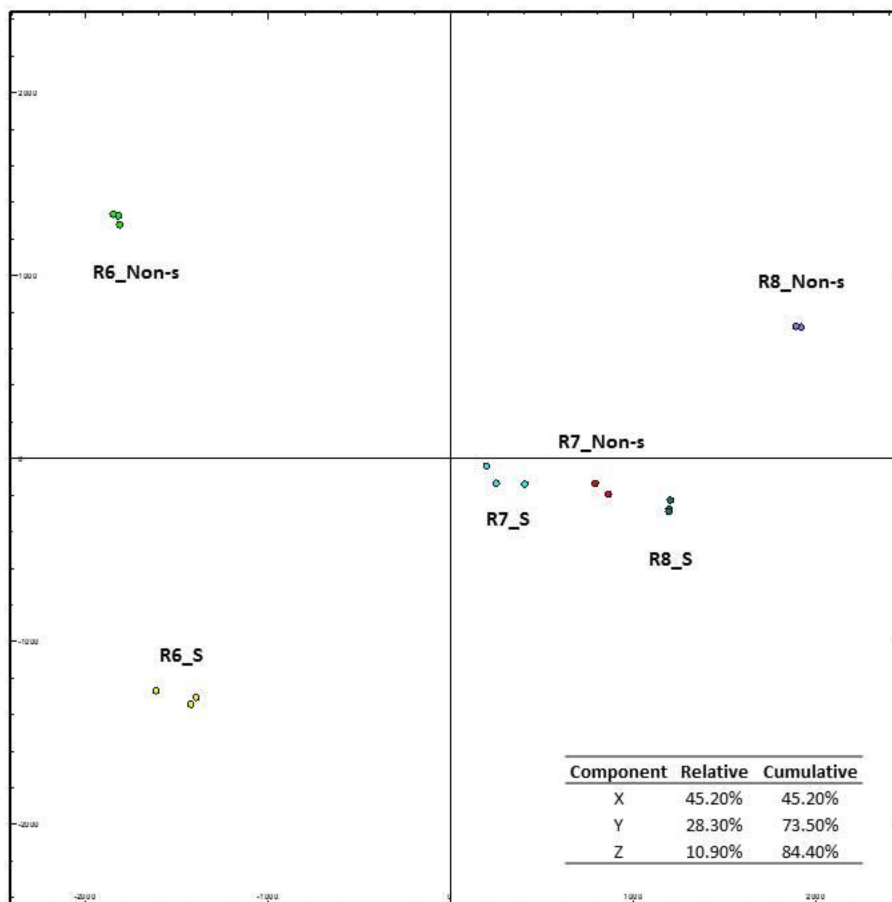


Figure 13. Principal component analysis of differentially expressed genes ($p \leq 0.05$ and $FC > 2$) in each stage of maturation. R6, R7 and R8 = stages of maturation; Non-S = non-stressed; S = stressed.

The 19,352 probesets were clustered to define 9 dominant expression patterns (DPs) (Figure 14), in order to give us an overview of gene expression changes during maturation of non-stressed and stressed seeds of the susceptible cultivar.

The mRNAs assigned to each pattern were classified in GO categories and an over representation analysis (ORA) was performed. This analysis compares a gene set of interest to a reference set and when considering a certain functional category as a GO term, it attempts to detect if this category is over-represented or under-represented in the respective gene set. It also estimates the likelihood of finding such numbers by chance (MAIA et al., 2011). The filtered gene set was used to determine GO categories that were significantly enriched (p-value ≤ 0.05) in the stressed/non-stressed seeds in the stages of maturation R6-R7-R8. The DPs 2, 3, 5 and 7 contain genes related to

the maturation process with only slight changes in expression for stressed seeds. The top 10 enriched GOs in each of the DPs, except for DP1 and 9 (that didn't have genes in significant ontologies) can be seen in Figure 15.

The genes clustered in DP 4 are more expressed in stressed seeds especially in R7. There are no unique enriched GOs for this pattern when compared to the four DPs mentioned before.

The expression of genes in DPs 1, 6, 8 and 9 (Figure 14) was more differentiated between non-stressed and stressed seeds which indicates that the genes assigned to these patterns are more likely to be related to stress than to changes due to seed maturation.

The expression of genes in DP 1 was higher for stressed seeds in all stages of maturation and an opposite pattern was observed compared to non-stressed seeds. Although 97 probe sets were assigned to this pattern, most of them are not annotated or refer to predicted uncharacterized proteins.

For the differential expression analysis of stressed and non-stressed seeds, genes were selected if their expression exhibited at least a 2-fold change in expression, comparing stressed with non-stressed seeds, and if the change in expression was statistically significant (p-value ≤ 0.05) over three biological replicates. After the first filtering step, in an individual comparison between stressed and non-stressed seeds for each stage of maturation (stressed – non-stressed in R6, R7 and R8), 672 transcripts were differentially expressed in stage R6, 513 transcripts in R7 and 265 transcripts in R8 (Figure 16).

In order to visualize the overall differentially expressed genes (p-value ≤ 0.05), the seed specific pathway of the Page-Man/MapMan package (http://MapMan.gabipd.org) was used. This pathway efficiently captures the most relevant molecular processes in seeds (Figure 17) (JOOSEN et al., 2011) and it allows a global overview of the ontologies of the up- and down-regulated genes in stressed seeds, during the maturation process.

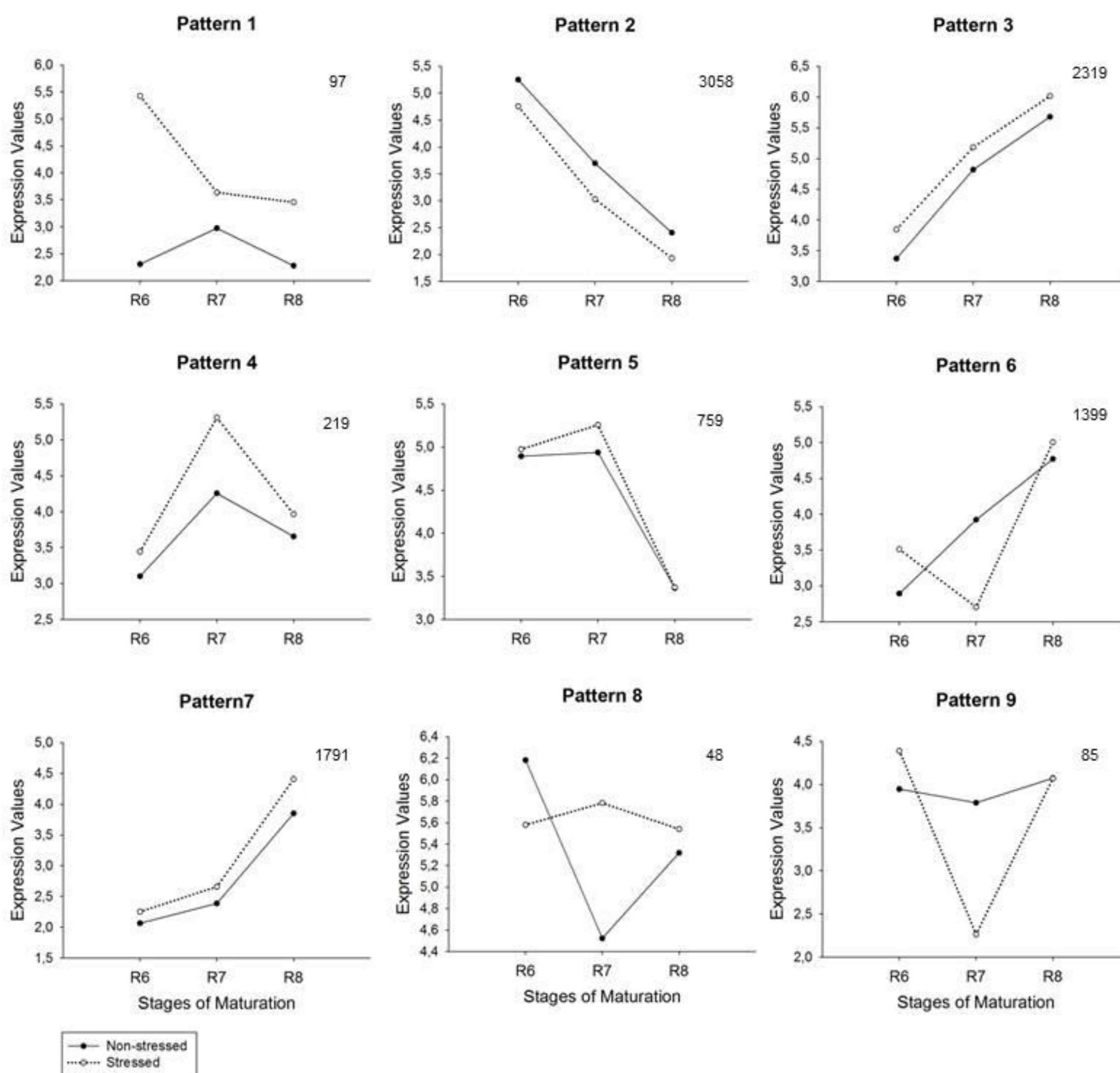


Figure 14. Dominant patterns (DPs) of gene expression in stressed and non-stressed soybean seeds of the susceptible cultivar in three stages of maturation (R6-R7-R8). Numbers at the top right corner represents the number of genes assigned to each DP.

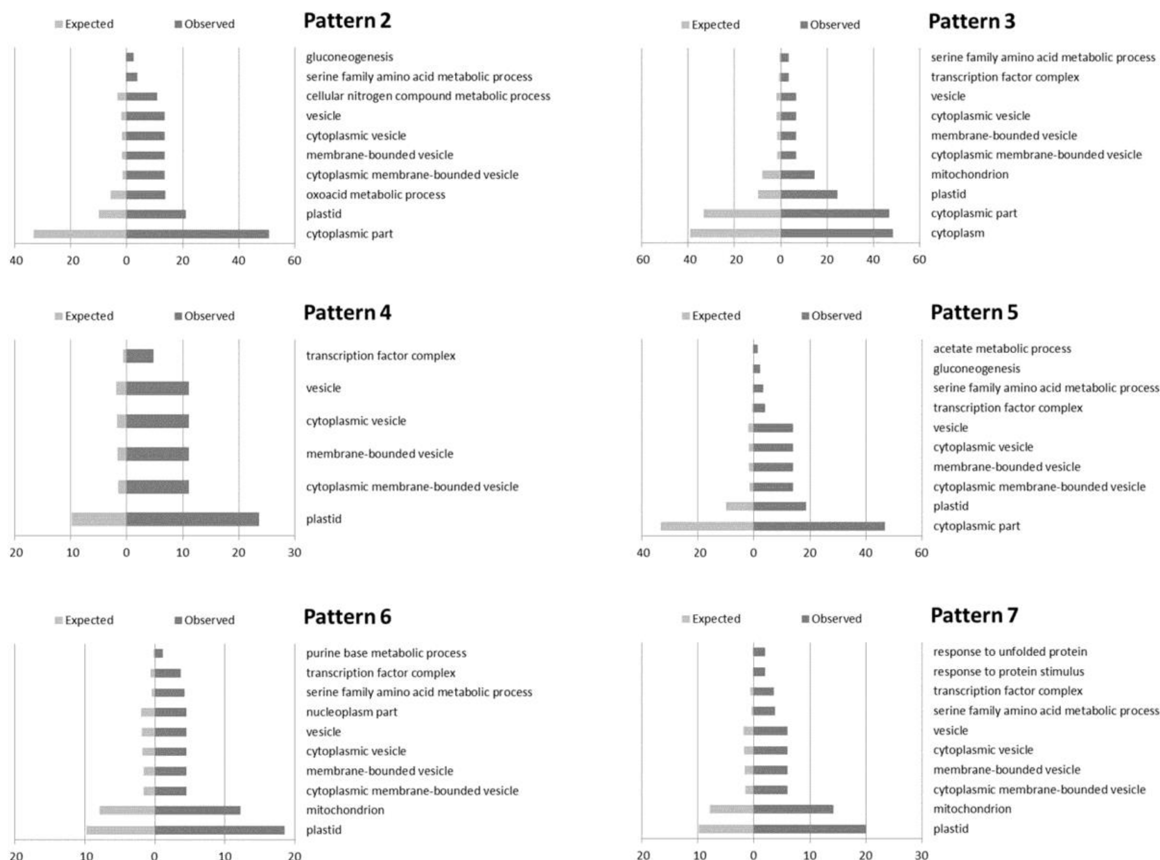


Figure 15. Top 10 enriched gene ontologies of the DPs 2, 3, 4, 5, 6, 7. GOs of DPs 1 and 9 are not shown due to the lack of genes significantly assigned to a GO.

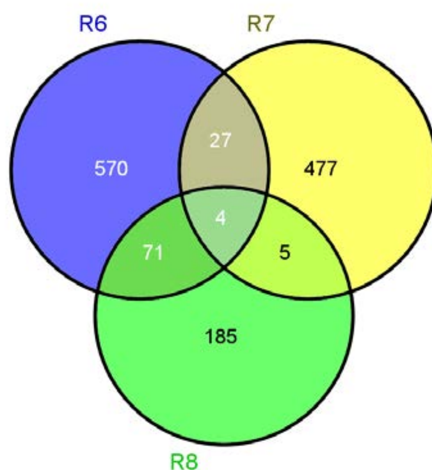


Figure 16. Venn diagram of the output of differentially expressed genes in each stage of maturation (R6-R7-R8).

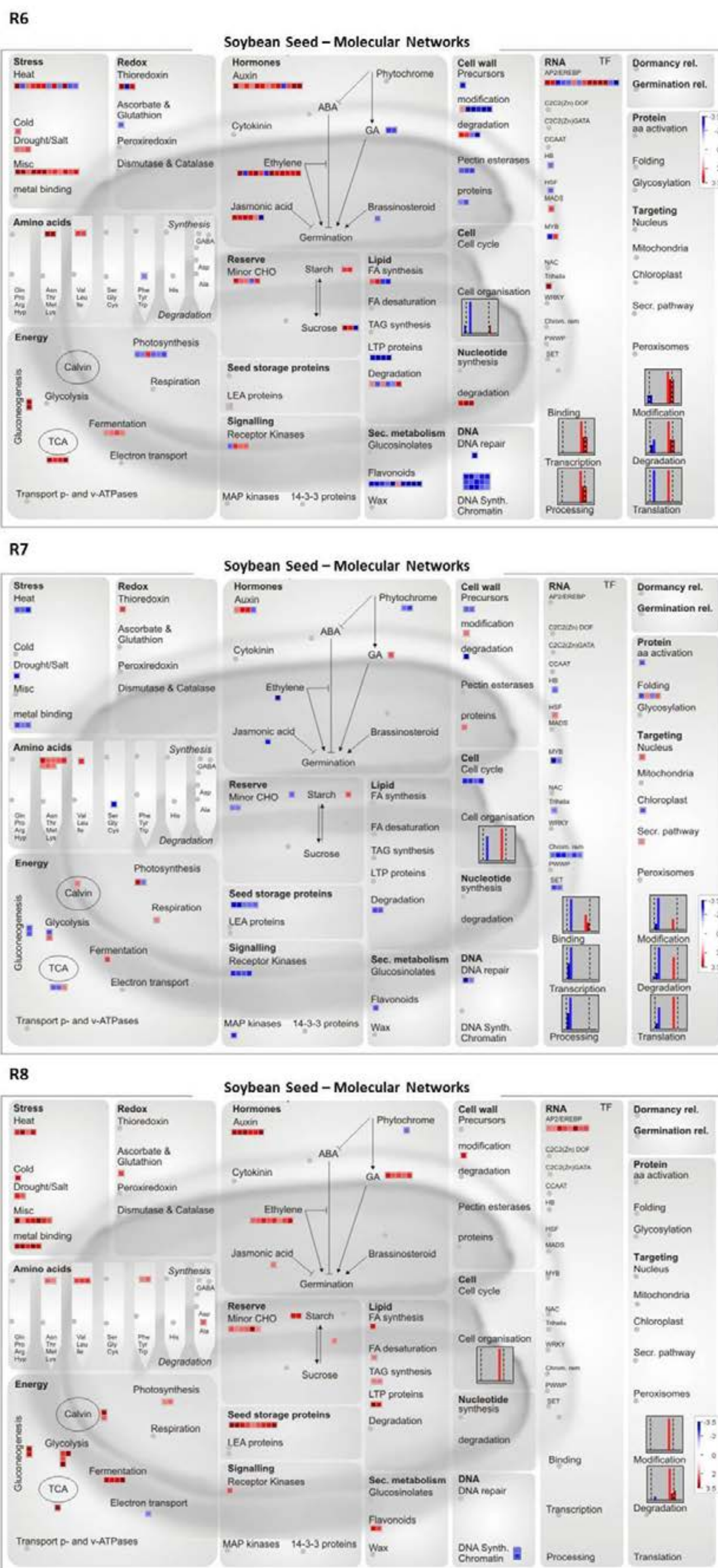


Figure 17. Seed MapMan molecular network map. Log_2 ratios are used to express relative transcript levels in stressed versus non-stressed soybean seeds in each stage of

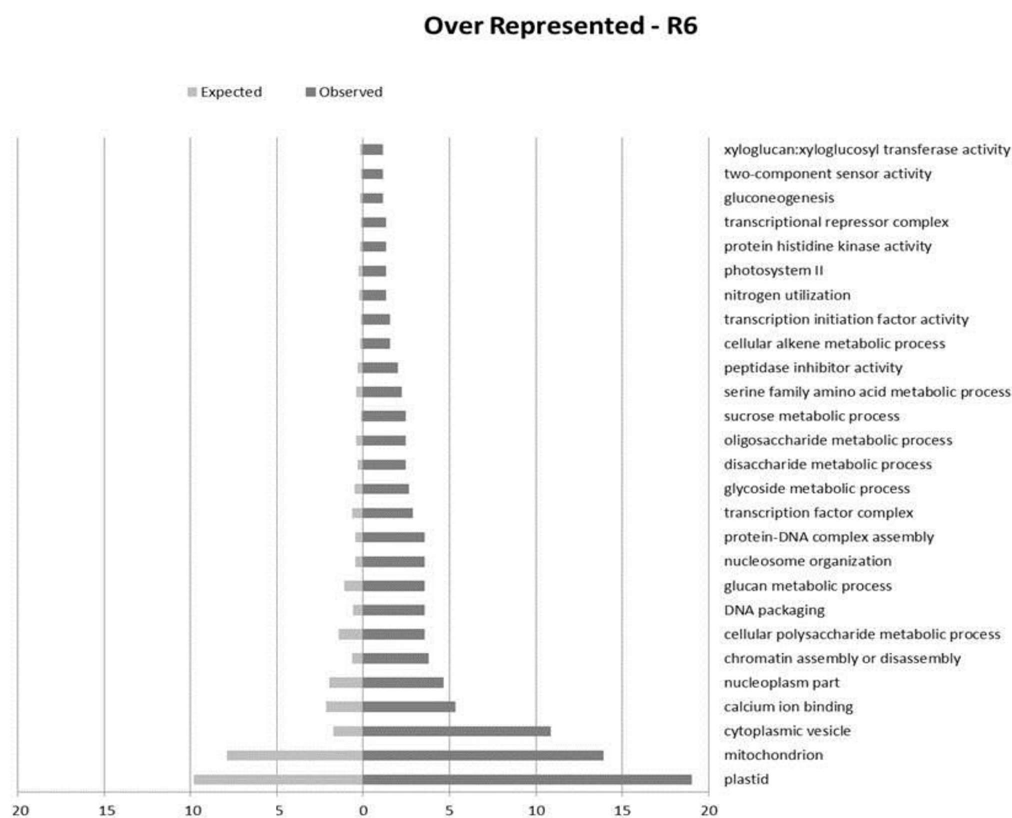
maturation (R6-R7-R8). Red squares depict higher levels in stressed seeds; blue squares higher levels in non-stressed seeds. Only ratios with P values lower or equal to 0.05 are displayed.

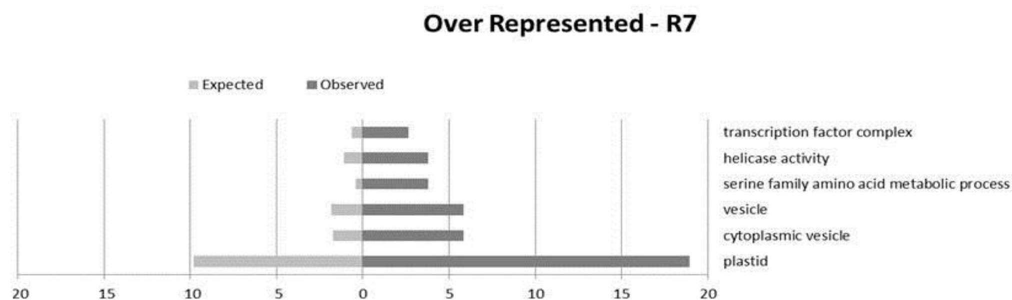
Multiple pathways were enriched in stressed seeds mainly in the stages R6 and R8. In R6 most of the genes in categories such as "stress", "hormones", "reserves", "energy metabolism" and "RNA" were up-regulated in the stressed seeds. In R8 pretty much all of the categories represented by the MapMan seed molecular network included genes up-regulated in stressed seeds, with a significant increase in seed storage protein genes up regulated in the stressed condition when compared with the previous stages. In R7 most of the genes in the different ontologies were up-regulated in non-stressed seeds, except for genes related to amino acid metabolism.

In order to verify in more detail how each metabolic pathway was enriched at each stage of maturation, an ORA was performed (Figure 18).

When stressed versus non-stressed seeds at each stage of maturation were analyzed individually, GOs such as "plastid", "cytoplasmic vesicle", "vesicle", "peptidase inhibitor activity", "serine family" and "aminoacid metabolic process" were common to the three stages of maturation. Many GOs were enriched only early in maturation (R6), for example the ones related to "nucleosome assembly", "chromatin and DNA packing", "transcription factor activity", "photosystem II" and "enzyme activity". Following the progress of maturation the number of significant enriched GOs was reduced. Only the "helicase activity" category was exclusive for R7 and "oxireductase activity", "enzyme inhibitor activity" and "nutrient reservoir activity" were important ontologies significantly enriched due to stress in R8.

As mentioned above (Figure 13) the differences in maturity within seeds in R7 can cause differences in response to stress and may have been the reason why gene expression at this stage is not resolved. Likewise this might explain the fewer ontologies specific to R7 observed in Figure 18.





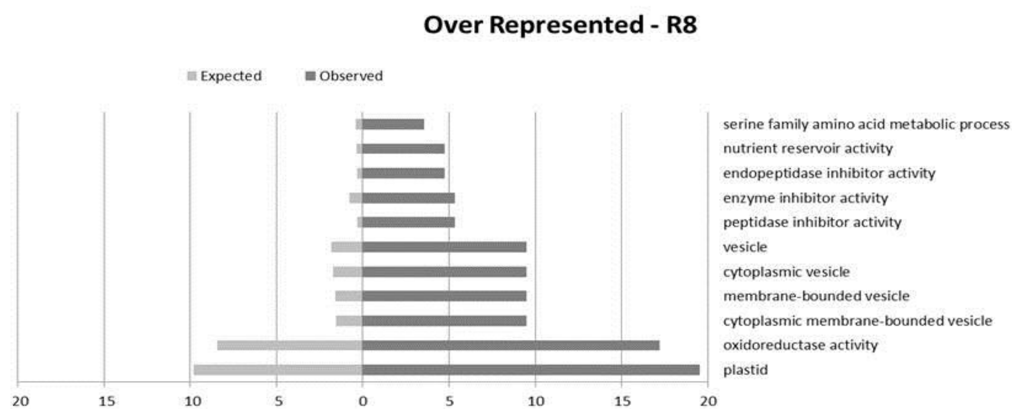


Figure 18. Over-representation analysis of the differentially expressed genes in stressed soybean seeds in three stages of maturation (R6-R7-R8). The gene set analyzed

was first filtered by a fold-change and variance (fold change > 2 and p-value ≤ 0.05). The bars are ordered on the observed/expected genes ratios.

Considering the uncertainty about R7, a new differential expression analysis was performed, not considering this stage, in order to find good candidate genes related to the Chl retention in soybean seeds. Genes were now selected if their expression exhibited at least a 2-fold change, and if the change in expression was statistically significant (p-value ≤ 0.05) over three biological replicates of stressed and non-stressed seeds during the maturation, from R6 to R8. Thus, the analysis was performed with the gene set of the comparison between stressed and non-stressed seeds considering also changes in expression during the maturation process from R6 to R8 (avoiding R7 data) [R6-R8 = (stressed R8 – stressed R6) – (non-stressed R8 – non-stressed R6)].

This analysis generated a set of 833 differentially expressed transcripts that was analyzed with the seed-specific pathway of the MapMan tool (Figure 19).

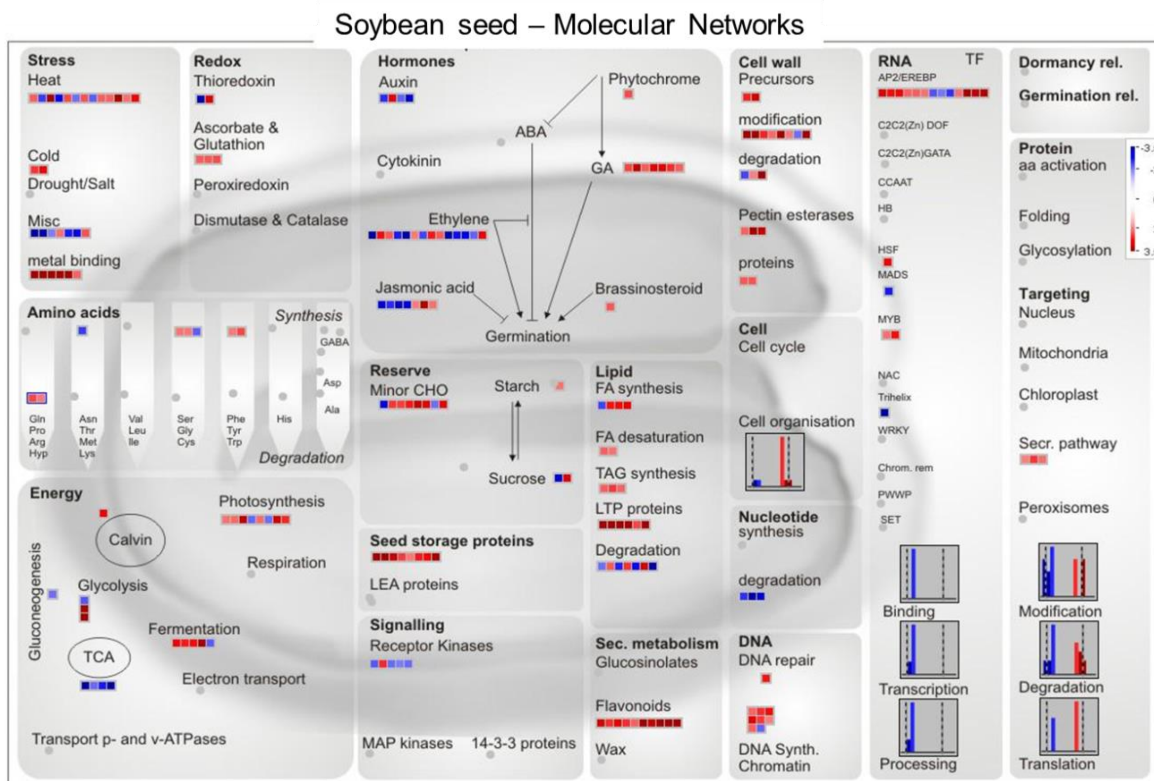


Figure 19. Seed MapMan molecular network map. Log2 ratios are used to express relative transcript levels in stressed versus non-stressed soybean seeds during maturation (R6-R8). Red squares depict higher levels in stressed seeds; blue

squares higher levels in non-stressed seeds. Only ratios with P values lower or equal to 0.05 are displayed.

Multiple pathways were enriched. As was expected, the stress affected the expression of genes related to a large number of different cell functions. As an example, photosynthesis, transcription factors such as AP2/EREBP, minor CHO (reserve), DNA repair/synthesis and cell wall modification, were GOs with genes mostly up-regulated in stressed seeds, whereas genes related to seed storage proteins, flavonoids (secondary metabolism), lipid transfer proteins and GA metabolism were only significantly up-regulated in stressed seeds.

APETALA2 (AP2) and ethylene-responsive element binding proteins (EREBPs) are members of a family of transcription factors unique to plants, whose distinguishing characteristic is that they contain the so-called AP2 DNA-binding domain. AP2/EREBP genes form a large multigene family, and they play a variety of roles throughout the plant life cycle: from being key regulators of several developmental processes, like floral organ identity determination or control of leaf epidermal cell identity, to forming part of the mechanisms used by plants to respond to various types of biotic and environmental stresses (RIECHMANN; MEYEROWITZ, 1998). AP2/EREBP transcription factors have been implicated in hormone, sugar and redox signaling in context of abiotic stresses such as cold and drought. Furthermore, members of the AP2/EREBP family are implicated in integration of signals derived from organelles in retrograde feedback loops and in stress acclimation (DIETZ et al., 2010).

Other hormone categories such as auxins, ethylene and jasmonic acid were also enriched in stressed seeds. These GOs are represented by different genes being up- and down-regulated. Although phytohormones affect a wide range of phenomena in plant metabolism, Kusaba et al. (2013) suggested that those groups of hormones can play roles in leaf senescence and even in the stay-green phenotype in some plant species.

Jasmonic acid (JA) is a phytohormone that, among other things, is involved in wound response, disease resistance and senescence (HE et al., 2002). JA promotes leaf senescence during a dark treatment. Consistent with this characterization, a loss-of-function mutant of *COI1* encoding the co-receptor of JA and the antisense transgenic plant of 3-ketoacyl-CoA thiolase 2 (KAT2) involved in JA synthesis have a stay-green phenotype in response to a dark incubation (CASTILLO; LEÓN, 2008).

However, Schommer et al. (2008) reported that *coi1* did not exhibit a stay-green phenotype during natural senescence and that the JA-deficient mutants of allene oxide synthase (*aos*) and oxophytodienoate reductase3 (*opr3*) also did not have a stay-green phenotype in response to dark incubation (KUSABA et al., 2013). Thus, the causal relationship between delayed senescence and the level of JA is not completely clear.

Auxins are a class of phytohormones that play a critical role in development and growth of plants. Contradictory results have related overexpression and inhibition of auxin biosynthesis to the stay-green phenotype (KIM et al., 2011). Kusaba et al. (2013) stated that these contradictory results suggest that auxin promotes rather than delays leaf senescence.

Although we observed expression of genes associated with such hormones being enriched in stressed soybean seeds that clearly show Chl retention, better analysis of the expression of genes clustered into these GOs and a closer look at hormone levels is necessary.

The filtered gene set was used to determine GO categories that were significantly enriched (p-value ≤ 0.05) in the stressed/non-stressed seeds during the maturation progress (from R6 to R8) (Figure 20).

GOs such as “oxireductase activity”, “enzyme inhibitor activity”, “transcription factor complex”, “nutrient reservoir activity” “photosystem II” and “transcriptional repressor complex” were among the top-ranked enriched processes.

Chl molecules mainly occur bound to apoproteins in the chloroplasts. As a result, the Chl breakdown is intimately related with the deconstruction of the photosynthetic apparatus, especially the light harvesting complexes. As the aim was to find genes related to Chl retention in soybean seeds during maturation, focus was given to genes related to photosynthesis and to enzymes involved in the Chl degradation pathway. Thus, gene expression studies were performed by RT-qPCR analysis, comparing stressed and non-stressed seeds of two soybean cultivars: the susceptible and the tolerant in three stages of maturation (R6-R7-R8).

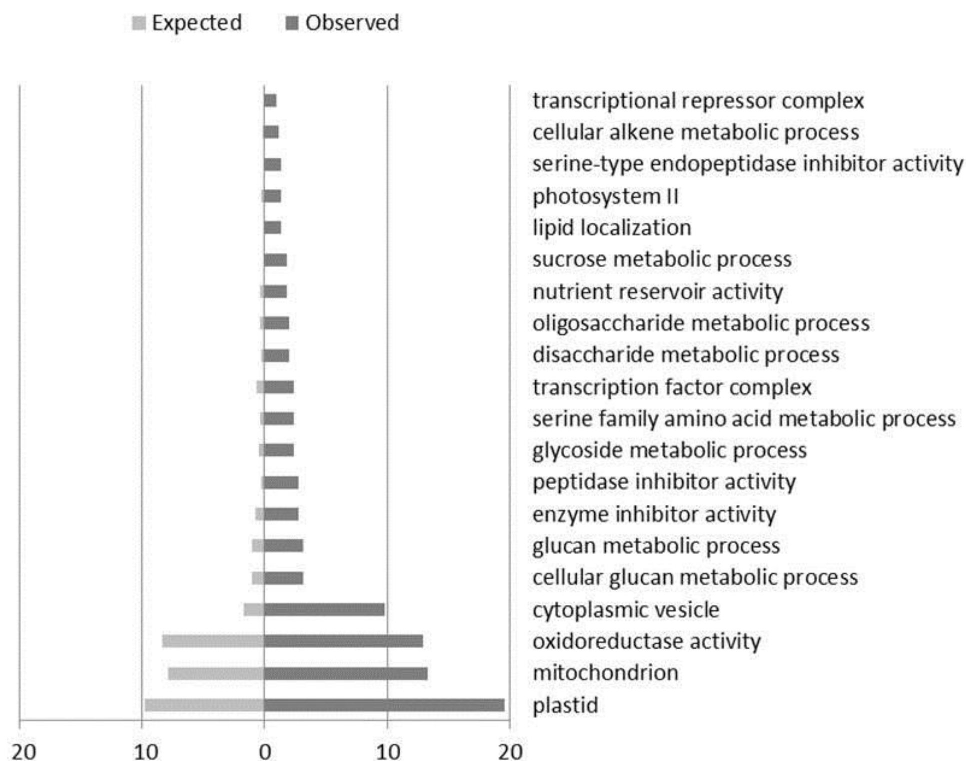


Figure 20. Over-representation analysis of the differentially expressed genes in stressed soybean seeds. The gene set analyzed was first filtered through a fold-change and variance cut-off based filter (fold change>2 and p-value <0.05). The bars are ordered on the observed/expected genes ratios.

4.5. Chlorophyll breakdown is impaired in stressed soybean seeds

Chl is essential for light capturing and it is the starting point of the process that provides the energy for photosynthesis and thus plant growth. In higher plants, there are two kinds of Chls, Chl a and Chl b, both of which can be bound by LHCI and LHCII, although Chl a can also bind to other subunits of PSI and PSII.

During leaf senescence the visible change is that green tissues turn yellow due to Chl degradation and breakdown of LHCI and LHCII. So far many studies have addressed senescence in leaves and a good model for this refers to the SGR mutants of different species. In contrast, very little is known about the Chl degradation process in developing and maturing seeds. Degreening in maturing seeds cannot be considered senescence but these processes share a common need for Chl degradation. To better

understand the breakdown of this pigment in soybean seeds and consequently its retention we analyzed the expression of some of the genes encoding for enzymes in the Chl degradation pathway. Among them are some that are known to cause the stay-green phenotype. For this analysis we compared stressed and non-stressed soybean seeds of the susceptible and tolerant cultivar during maturation (R6-R7-R8).

The first step in Chl degradation is the conversion of Chl b to Chl a, catalyzed by Chl b reductase. The expression of two transcripts encoding for this enzyme, *NYC1_1* (Glyma07g09430) and *NYC1_2* (Glyma09g32370) (Figure 21) was analyzed.

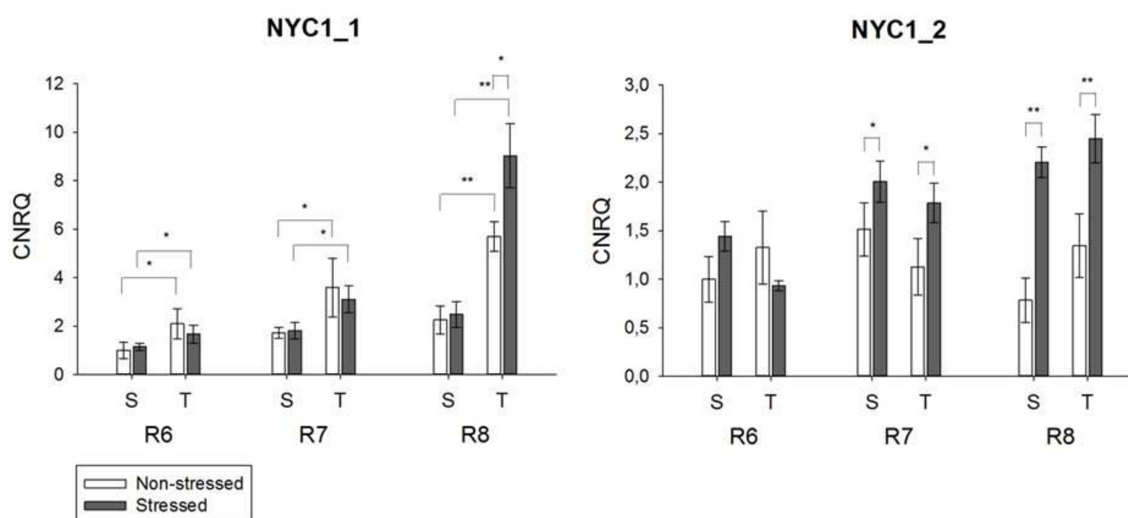


Figure 21. Gene expression analysis by real-time quantitative PCR (RT-qPCR) represented as the calibrated normalized relative quantity (CNRQ) for *NYC1_1* and *NYC1_2*. Gene expression is shown for non-stressed (open bars) and stressed seeds (closed bars) of the susceptible (S) and tolerant (T) cultivar in three stages of maturation (R6-R7-R8). Bars represent the mean values ± SE of three independent biological replicates. Bars connected by brackets represent the statistical comparisons. Asterisks represent statistically significant differences (*, $P \leq 0.05$; **, $P \leq 0.01$).

The expression of *NYC1_1* in seeds of the susceptible cultivar increased very slightly during maturation, but there was no difference between stressed and non-stressed seeds. However, for the tolerant cultivar there was a clear increase in expression during maturation and, especially in R8, the expression was significantly higher in stressed seeds. The relative expression of *NYC1_2* was more constant during maturation of non-stressed seeds of both cultivars, but for stressed seeds the expression increased during maturation. For both cultivars there were higher levels of *NYC1_2* transcripts in stressed seeds in R7 and R8. As the amount of Chl b is higher in stressed seeds, it is expected that higher levels of Chl b reductase are available in these seeds to convert Chl b to Chl a and thus allow the continuity of the degradation flow. It seems that *NYC1_1* is playing a major role in seeds of the tolerant cultivar. This gene is more activated in this cultivar in both stressed and non-stressed seeds which could explain why seeds of this cultivar contain less Chl towards the end of the maturation process.

nyc1 mutants in rice have been described as defective in Chl b reductase synthesis (*NYC1* and *NOL*). Those mutants retain Chl in leaves during senescence, resulting in a stay-green phenotype (KUSABA et al., 2007, 2013; SATO et al., 2009).

Chl breakdown continues with the removal of Mg and phytol by metal-chelating substances (MCS) and pheophytinases/chlorophyllases, resulting in pheide a. Although four genes could be identified as chlorophyllases in soybean (Glyma10g00570, Glyma09g31250, Glyma07g10831, Glyma02g00770) the expression of three of them was undetectable. And therefore the expression of only one chlorophyllase gene (*CLH*) was analyzed

The relative expression of *CLH* (Glyma10g00570) analyzed here decreased during the maturation of non-stressed seeds of both cultivars. It remained relatively constant during the maturation of stressed seeds (Figure 22).

The transcript level of *CLH* was higher in stressed seeds of the susceptible cultivar in R7 and R8, but was higher in stressed seeds of the tolerant cultivar only in R8. Although the expression of this enzyme is higher in stressed seeds, that contain higher Chl amounts, the pigment remains visible and detectable until late in maturation mainly in stressed seeds of the susceptible cultivar. Judged from the expression pattern of this gene, there is no difference that could explain why one cultivar retains Chl in the seed and the other does not.

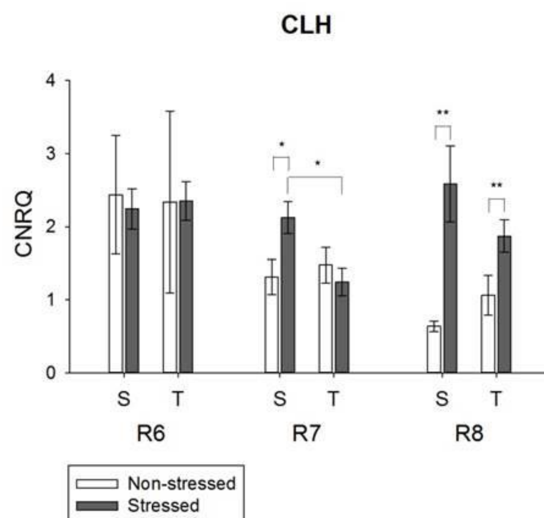


Figure 22. Gene expression analysis by real-time quantitative PCR (RT-qPCR) represented as the calibrated normalized relative quantity (CNRQ) for *CLH*. Gene expression is shown for non-stressed (open bars) and stressed seeds (closed bars) of the susceptible (S) and tolerant (T) cultivars in three stages of maturation (R6-R7-R8). Bars represent the mean values ± SE of three independent biological replicates. Bars connected by brackets represent the statistical comparisons. Asterisks represent statistically significant differences (*, $P \leq 0.05$; **, $P \leq 0.01$).

Chlorophyllase was already demonstrated as not being crucial for Chl degradation in leaves; hence, the double mutant of *AtCLH1* and *AtCLH2* in Arabidopsis did not generate a stay-green phenotype (SCHENK et al., 2007). This finding suggested that chlorophyllase might not play a major role in Chl degradation during leaf senescence (SCHENK et al., 2007; SATO et al., 2009). Instead, the functional characterization of PPH has indicated that this enzyme is necessary for Chl breakdown in Arabidopsis (SCHELBERT et al., 2009) and rice (SATO et al., 2009). We suggest here that this is also the case for Chl degradation during soybean seed degreening based on the

expression of three *PPH* genes in stressed and non-stressed seeds during the maturation progress (Figure 23).

The expression of *PPH1* (Glyma09g36010) and *PPH3* (Glyma12g01320) decreased during maturation, while *PPH2* (Glyma11g16070) increased. *PPH1* and *3* seem to be required earlier in maturation for the progress of Chl degradation. The expression of both genes in R6 was lower in stressed seeds of both cultivars, although it was significantly higher in stressed and non-stressed seeds of the tolerant one when compared with the susceptible cultivar. In R7, the expression of *PPH*3 wasn't affected by the environmental condition or cultivar, but the expression of *PPH1* was higher in stressed and non-stressed seeds of the tolerant cultivar. In R8, the expression of *PPH1* was not detected and for *PPH3* it was lower in stressed seeds of both cultivars.

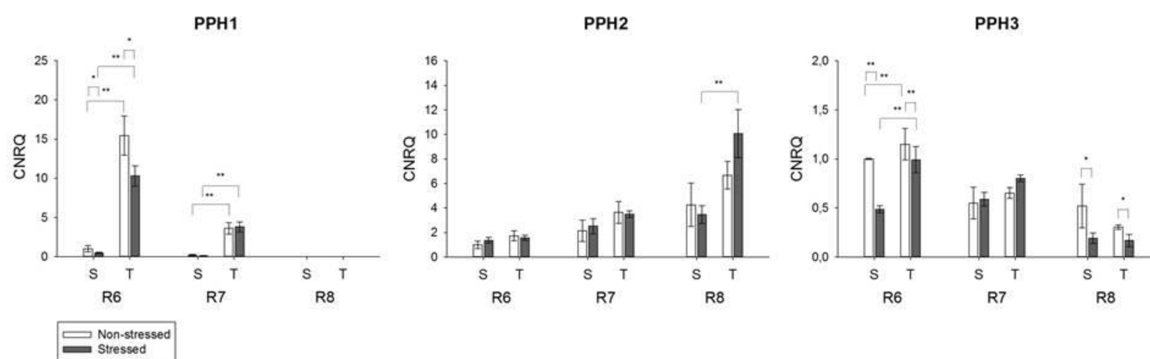


Figure 23. Gene expression analysis by real-time quantitative PCR (RT-qPCR) represented as the calibrated normalized relative quantity (CNRQ) for PPH1, PPH2 and PPH3. Gene expression is shown for non-stressed (open bars) and stressed seeds (closed bars) of the susceptible (S) and tolerant (T) cultivars in three stages of maturation (R6-R7-R8). Bars represent the mean values ± SE of three independent biological replicates. Bars connected by brackets represent the statistical comparisons. Asterisks represent statistically significant differences (*, $P \leq 0.05$; **, $P \leq 0.01$).

PPH2 expression seems to be required later in maturation, especially in stressed seeds of the tolerant cultivar. This specific gene might be a key for Chl degradation in seeds. Its expression was significantly higher (≈ 7X) in stressed seeds of the tolerant cultivar than in stressed seeds of the susceptible one.

Phein a, an intermediate of *PPH* catalysis, is known to be accumulated in the *pph* mutant in Arabidopsis and in the *nyc3* mutant in rice (SCHELBERT et al., 2009; MORITA et al., 2009). The phein a content in other stay-green mutants has not been reported (KUSABA et al., 2013). Although we didn't measure the accumulation of phein a in this study we suggest that it might be occurring in stressed seeds of the susceptible cultivar and thereby being responsible, together with Chl a and b accumulation, for the green color observed in mature seeds produced under stressful conditions.

In the *pph* mutant mentioned above, phein a accumulates to a slightly greater amount than in wild-type plants and the rate of Chl breakdown in the *pph* mutant appeared to slow down indicating that excess of phein a accumulation suppresses Chl breakdown activity via a negative feedback loop (SCHELBERT et al., 2009; MORITA et al., 2009).

Downstream of the degradation pathway, after phein a is converted by *PPH* to pheide a, this intermediate is subsequently converted to the red-colored molecule RCC in a reaction mediated by PAO. The expression of two genes encoding for PAO was analyzed: *PAO1* (Glyma11g19800) and *PAO2* (Glyma12g08740). The expression of both genes was higher in stressed seeds of the susceptible and tolerant cultivars mainly in R8, but the expression of *PAO1* was always higher in stressed and non-stressed seeds of the tolerant cultivar in comparison with the seeds of the susceptible cultivar in the respective conditions (Figure 24).

Although we did not measure the accumulation of pheide a and its oxidative damage, its accumulation is probably occurring in stressed seeds of the susceptible cultivar and could be acting as a negative feedback in other steps of the degradation pathway. For example, the non-yellowing *senescence-induced-deficiency* (*sid)* mutant of *Festuca. pratensis* accumulates significant amounts of chlorophyllide a and pheide a in the senescing leaves and has no PAO activity (VICENTINI et al., 1995; ROCA

et al., 2004), suggesting that Chl dephytylation by chlorophyllases is suppressed by pheide a accumulation in senescing leaves (PARK et al., 2007). Another feedback mechanism is presented in studies with the PAO-deficient mutants *pao1* and *acd1* (PARK et al., 2007). In these mutants SGR expression is severely inhibited probably through the accumulation of pheide a, which prevents further apoprotein disassembly and thus further Chl breakdown (HÖRTENSTEINER, 2009; SAKURABA et al., 2012).

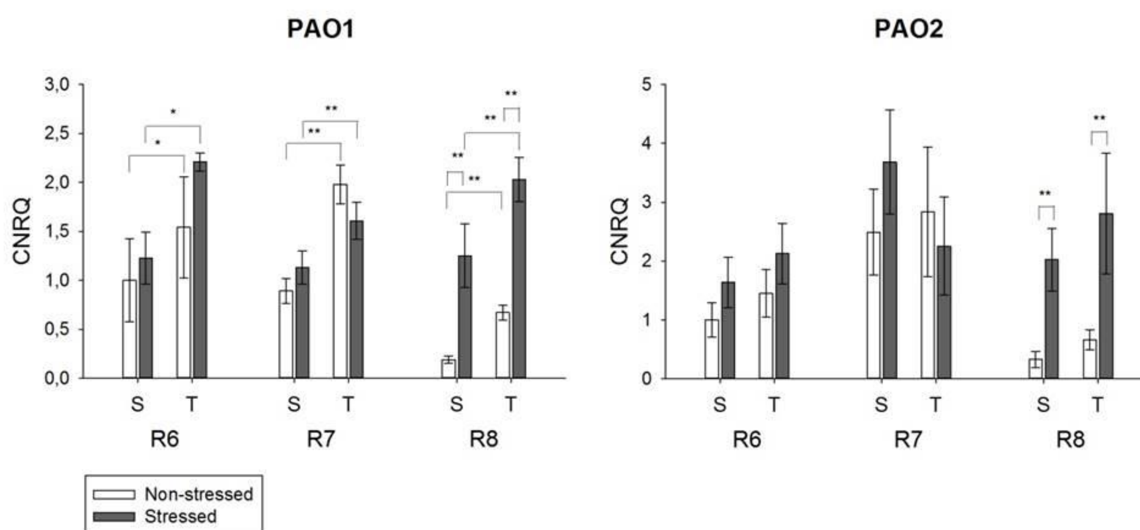


Figure 24. Gene expression analysis by real-time quantitative PCR (RT-qPCR) represented as the calibrated normalized relative quantity (CNRQ) for *PAO1* and *PAO2*. Gene expression is shown for non-stressed (open bars) and stressed seeds (closed bars) of the susceptible (S) and tolerant (T) cultivars in three stages of maturation (R6-R7-R8). Bars represent the mean values ± SE of three independent biological replicates. Bars connected by brackets represent the statistical comparisons. Asterisks represent statistically significant differences (*, $P \leq 0.05$; **, $P \leq 0.01$).

However, there are contradictory results available in literature. Sakuraba et al. (2013) affirmed that it is unlikely that lack of PAO, or pheide a accumulation regulates Chl breakdown by feedback. As in the Arabidopsis *hcar* mutant,

that has reduced PAO activity, a substantial number of leaf cells are dead (HIRASHIMA et al., 2009), it is likely that the plants only look green due to the color of pheide a. Yet, to better understand this feedback control, or the cause of green color in stressed soybean seeds, further analysis of PAO activity and the accumulation of intermediates of the pathway should be performed.

In the next step of Chl degradation, the red pigment (RCC) is converted to the non-colored but blue-fluorescing product pFCC by the RCCR. The pFCC will be further converted to NCCs through different mechanisms depending on the species. The expression of two genes encoding RCCR in soybean was quite different during seed maturation (Figure 25). This difference in expression between two genes encoding for the same enzyme was also observed for some of the genes discussed above, nevertheless it is not uncommon. Soybean has undergone at least two polyploidy or whole genome duplication events within the last 60 million years (SHOEMAKER et al., 2006; SCHLUETER et al., 2007; CHAN et al., 2012). As a result, approximately 75% of the genes are present in more than one copy in the soybean genome (SCHMUTZ et al., 2010), although it is hypothesized that functions of some duplicated gene pairs are highly diverged (FANG et al., 2014).

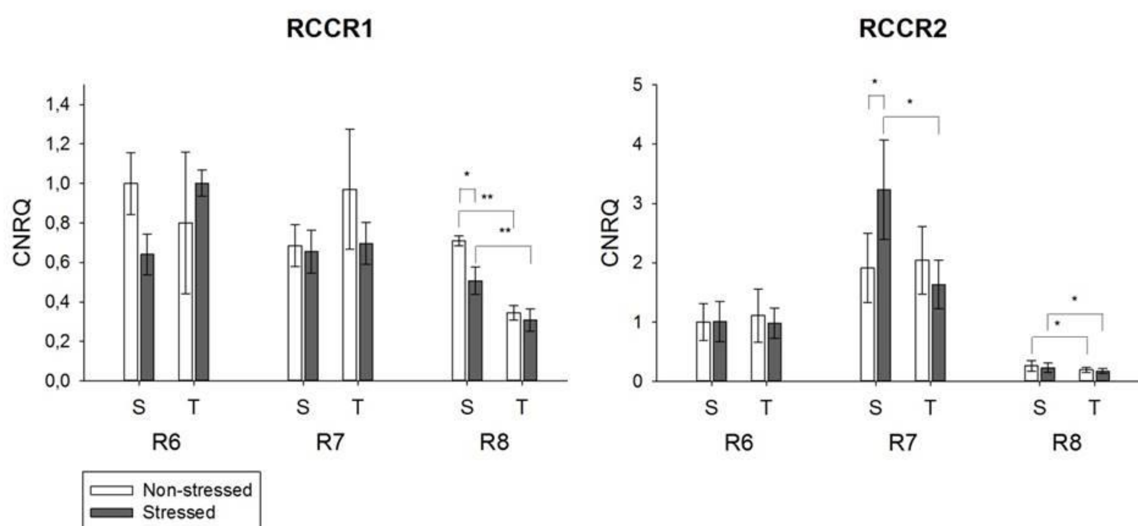


Figure 25. Gene expression analysis by real-time quantitative PCR (RT-qPCR) represented as the calibrated normalized relative quantity (CNRQ) for *RCCR1*

and *RCCR2*. Gene expression is shown for non-stressed (open bars) and stressed seeds (closed bars) of the susceptible (S) and tolerant (T) cultivars in three stages of maturation (R6-R7-R8). Bars represent the mean values ± SE of three independent biological replicates. Bars connected by brackets represent the statistical comparisons. Asterisks represent statistically significant differences (*, $P \leq 0.05$; **, $P \leq 0.01$).

The expression of *RCCR1* (Glyma14g01620) in R8 was lower in stressed than non-stressed seeds of the susceptible cultivar. Also the level of expression was lower for both stressed and non-stressed seeds of the tolerant cultivar when compared with seeds of the susceptible one produced in the respective environmental conditions. The difference in expression of *RCCR2* (Glyma02g47120) was already significant in R7. In this stage of maturation the effect of the environmental condition was dependent on the cultivar, thus the expression of *RCCR2* in stressed seeds of the susceptible cultivar was higher but there was no difference between stressed and non-stressed seeds of the tolerant cultivar. Also, the effect of cultivar was dependent on the environmental condition, so the expression was higher in stressed seed of the susceptible cultivar than in stressed seeds of the tolerant cultivar (Figure 25). In R8, the expression was not affected by the environmental condition, but it was higher in stressed and non-stressed seeds of the susceptible cultivar.

Pružinská et al.(2007) reported that the Arabidopsis *acd2-2* mutant, lacking RCC reductase, did not show a stay-green phenotype. These data indicate that the lack of RCC reductase does not have to affect overall Chl breakdown. Whether or not mutants with alterations further downstream in the Chl degradation pathway from RCC exhibit stay-green phenotypes has not been determined yet (SAKURABA et al., 2013). Deficiency in PAO or RCCR results in an accelerated cell death phenotype, which is caused by the accumulation of the substrates of the respective reactions, pheide a or RCC (MACH et al., 2001; PRUZINSKÁ et al., 2003; PRUŽINSKÁ et al., 2005, 2007). These colored intermediates of Chl breakdown are potentially phototoxic, and tight control of the PAO pathway has been considered important to prevent premature cell death during senescence (HÖRTENSTEINER, 2006).

Sgr is a nuclear gene encoding a chloroplast protein, and its homologs exist as either single or duplicated genes in higher plants (PARK et al., 2007). Fang et al (2014) described *D1* and *D2* in soybean as two unlinked, paralogous nuclear genes, whose double-recessive mutant (*d1d1d2d2*) results in Chl retention in leaves and seeds. We analyzed the expression of *D1* and *D2* in stressed and non-stressed seeds of the susceptible and tolerant cultivar (Figure 26).

The expression of both genes increased quite considerably during the progression of seed maturation, showing the highest levels at R8. However, *D2* seems to play a more important role during seed degreening as its levels of relative expression at all stages of maturation were higher than the levels of *D1*. The expression of *D1* in R7 was higher in stressed and non-stressed seeds of the tolerant cultivar compared to the susceptible one, but the expression of *D2* in R7 was not affected at all by environmental conditions or cultivar. In R8 the expression of both genes was higher in stressed seeds of both cultivars and higher in seeds of the tolerant cultivar in general.

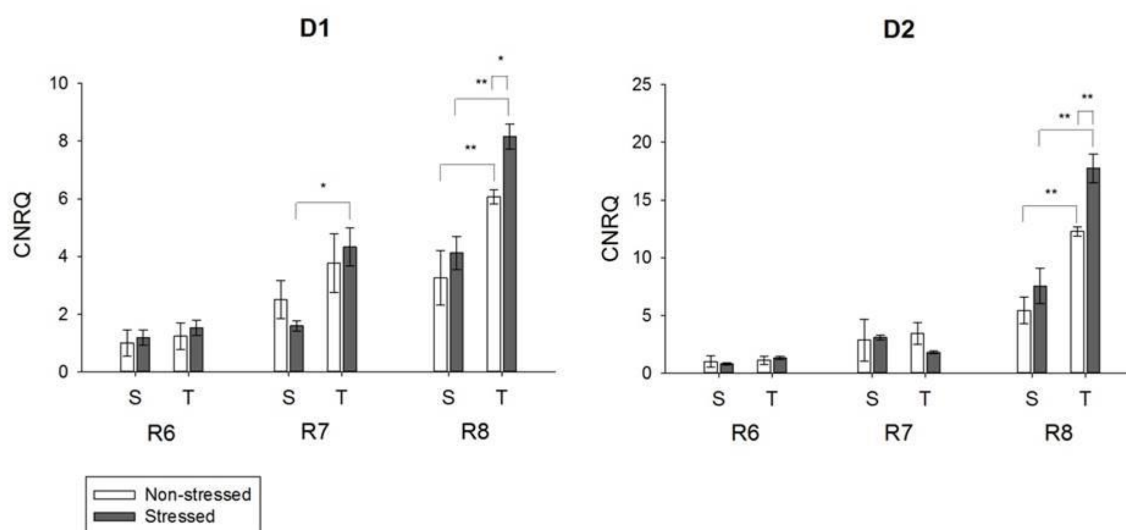


Figure 26. Gene expression analysis by real-time quantitative PCR (RT-qPCR) represented as the calibrated normalized relative quantity (CNRQ) for *D1* and *D2*. Gene expression is shown for non-stressed (open bars) and stressed seeds (closed bars) of the susceptible (S) and tolerant (T) cultivars in three stages of maturation (R6-R7-R8). Bars represent the mean values ± SE of three

independent biological replicates. Bars connected by brackets represent the statistical comparisons. Asterisks represent statistically significant differences (*, $P \leq 0.05$; **, $P \leq 0.01$).

Although the expression of *D1* and *D2* was higher in stressed seeds of both cultivars especially in R8, these genes were more expressed in stressed and non-stressed seeds of the tolerant cultivar that do not significantly retain Chl in stressful conditions. This difference in levels of expression of SGR between the two cultivars might be enough to maintain disassembly of the Chl-protein complexes and Chl degradation in the tolerant cultivar but not in the susceptible under heat and drought stress.

SGR transcription in rice is highly induced not only by the onset of senescence but also by developmental signals and environmental stresses, possibly in order to prevent Chl accumulation or activate Chl catabolism during development (PARK et al., 2007). Besides the developmental signals and environmental stresses regulating SGR genes as suggested above, an increase of pheide a or a decrease of *PAO* activity in the chloroplasts can act as negative feedback regulation of plastid to nucleus to repress SGR genes at the transcription level (PARK et al., 2007). The lower expression of *PAO* in seeds of the susceptible cultivar is possibly resulting in pheide a accumulation which, in agreement with the previous statement, would cause the lower expression of SGR (*D1/D2*) in seeds of this cultivar.

It is important to highlight that in R8 non-stressed seeds of both cultivars did not display detectable Chl, explaining the significant lower expression of all Chl catabolite enzymes (CCEs) and also of SGR in these seeds.

Apart from Chl retention, *sgr* mutants in various plants also retain large quantities of LHCII subunits (JIANG et al., 2007; PARK et al., 2007; AUBRY et al., 2008). This occurs because Chl breakdown is closely related to the degradation of Chl binding proteins. While the biochemical pathway of Chl breakdown is largely elucidated, little is known regarding proteases that might be involved in apoprotein degradation (HÖRTENSTEINER; FELLER, 2002).

4.6. The disassembly of photosystems

Since Chl mainly exists in association with proteins (Chl-protein complexes), degradation of this pigment during leaf senescence is coupled to degradation of the Chl-protein complexes found in the thylakoid membrane, including PSI, PSII and cytochrome B_6F (KUSABA et al., 2007; SATO et al., 2009; YAMATANI et al., 2013). Although we did not measure accumulation/degradation of the Chl-protein complexes, the expression of genes encoding for such proteins during the maturation process of stressed and non-stressed soybean seeds, can give us a clue of what happens with the photosystems and more specifically with the apoproteins that bind Chls.

The chosen transcripts represent genes encoding for proteins binding Chl (and other pigments), playing roles in photosystem I (*LHCA, psaA, psaB*), photosystem II (*psbA, psbB, psbC, psbD, psbE, psbF*) and cytochrome B_6F (*Cyt* B_6F).

The expression of cytochrome B_6F (*Cyt B6F*, Glyma12g32580) and light harvesting complex I (*LHCA*, Glyma16g26130) (Figure 27) decreased significantly during maturation.

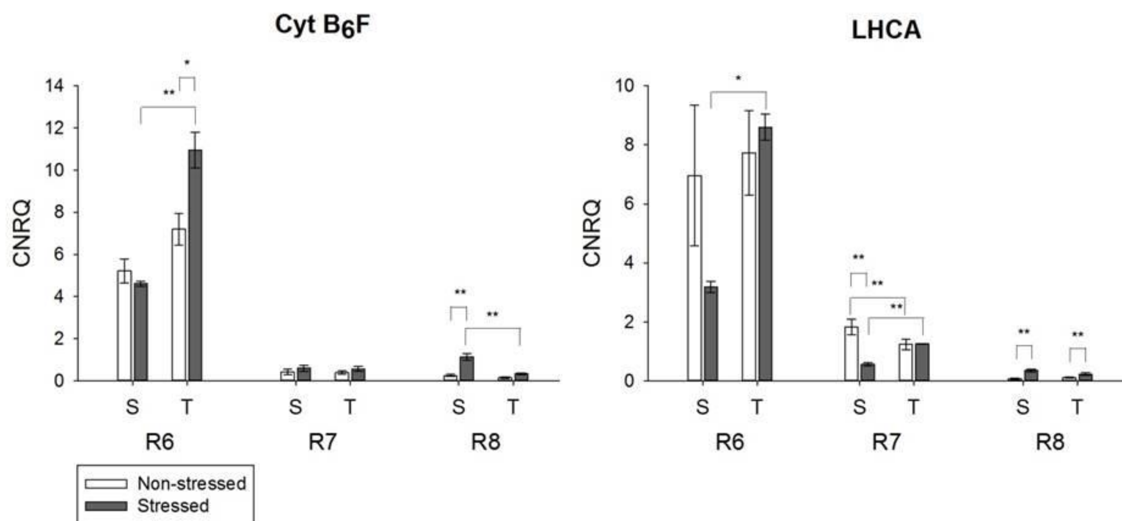


Figure 27. Gene expression analysis by real-time quantitative PCR (RT-qPCR) represented as the calibrated normalized relative quantity (CNRQ) for *Cyt* B_6F and *LCHA*. Gene expression is shown for non-stressed (open bars) and stressed seeds (closed bars) of the susceptible (S) and tolerant (T) cultivars in three

stages of maturation (R6-R7-R8). Bars represent the mean values ± SE of three independent biological replicates. Bars connected by brackets represent the statistical comparisons. Asterisks represent statistically significant differences (*, $P \leq 0.05$; **, $P \leq 0.01$).

The expression of *Cyt B_6F* was higher in stressed seeds of the tolerant cultivar in R6, but in R8 stressed seeds of the susceptible cultivar presented higher levels of this transcript. Although the expression of *LHCA* was higher in stressed than non-stressed seeds of both cultivars late in maturation (R8), it was relatively low compared with the previous stages of maturation. This decrease in expression of both genes along the maturation process is probably related to the degradation of most of the Chl and consequently of the apoproteins binding the pigment. The disassembly of the LHCs, especially LHCII, is crucial for degreening during leaf senescence, as these peripheral antennas contain most of the Chl. LHCII is the most abundant pigment-protein complex, binding Chl a and b, which account for 40% of the total Chl (TANAKA et al., 1987; CONSOLI et al., 2005; HORIE et al., 2009). The retention of LHCII has been associated with the stay-green phenotype in some species (GUIAMÉT et al., 1991; KUSABA et al., 2007; SAKURABA et al., 2012; GRASSL et al., 2012; HÖRTENSTEINER, 2013). Despite the importance of LHCII degradation to the degreening process, the expression of the gene encoding this protein (*LHCB*) was not analyzed in this study.

The expression of the inner antennas *psbB* (CP47) and *psbC* (CP43) of PSII are shown in Figure 28.

The expression of *psbB* decreased during maturation of stressed and non-stressed seeds of the susceptible and tolerant cultivar. At R7 the expression of psbB was higher in stressed seeds of both cultivars, but the levels of transcript was higher in stressed and non-stressed seeds of the tolerant cultivar when compared with stressed and non-stressed seeds of the susceptible cultivar. In R8, the difference in expression between stressed and non-stressed seeds was significant only for the susceptible cultivar. This suggests that the variation in the expression of this gene late in maturation may be dependent on the genetic material.

The expression of *psbC* decreased slightly during the maturation of seeds of the susceptible cultivar, but the decrease was greater for the tolerant one. In all stages of maturation the expression of *psbC* was higher in both stressed and non-stressed

seeds of the tolerant cultivar. In R7 and R8, for both cultivars, the expression of this gene was higher in stressed seeds.

The expression of *psbA* and *psbD,* genes encoding for the core proteins of PSII (D1and D2 proteins, respectively) was also analyzed (Figure 29).

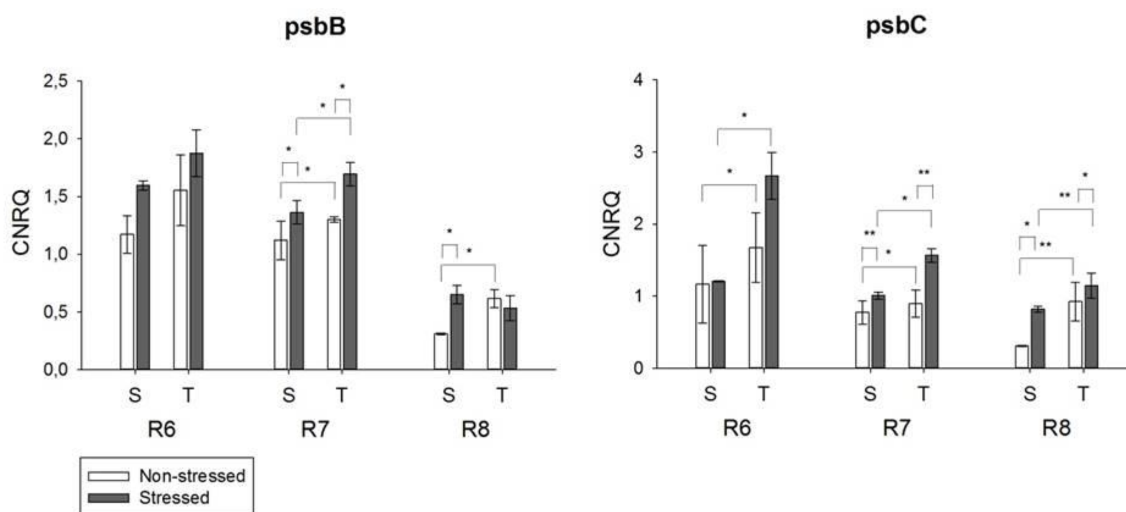


Figure 28. Gene expression analysis by real-time quantitative PCR (RT-qPCR) represented as the calibrated normalized relative quantity (CNRQ) for *psbB* and *pcbC*. Gene expression is shown for non-stressed (open bars) and stressed seeds (closed bars) of the susceptible (S) and tolerant (T) cultivars in three stages of maturation (R6-R7-R8). Bars represent the mean values ± SE of three independent biological replicates. Bars connected by brackets represent the statistical comparisons. Asterisks represents statistically significant differences (*, $P \leq 0.05$; **, $P \leq 0.01$).

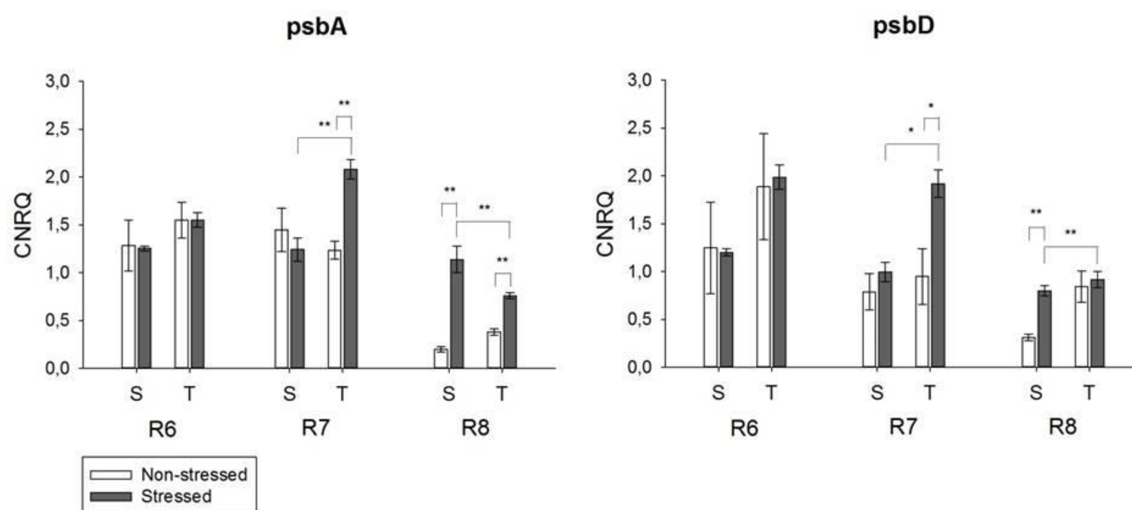


Figure 29. Gene expression analysis by real-time quantitative PCR (RT-qPCR) represented as the calibrated normalized relative quantity (CNRQ) for *psbA* and *psbD.* Gene expression is shown for non-stressed (open bars) and stressed seeds (closed bars) of the susceptible (S) and tolerant (T) cultivars in three stages of maturation (R6-R7-R8). Bars represent the mean values ± SE of three independent biological replicates. Bars connected by brackets represent the statistical comparisons. Asterisks represents statistically significant differences (*, $P \leq 0.05$; **, $P \leq 0.01$).

The expression of *psbA* and *psbD* in non-stressed seeds of both cultivars decreased during maturation. Stressed seeds of the tolerant cultivar had an increase in expression of *psbA* in R7 followed by a decrease in R8. In contrast, the expression of this gene in stressed seeds of the susceptible cultivar remained constant during the whole maturation period. Towards the end of maturation (R8), the expression of *psbA* was higher in stressed seeds of both cultivars, but was significantly higher in stressed seeds of the susceptible cultivar than in stressed seeds of the tolerant one. The expression of *psbD* followed similar patterns during maturation, however, in R8 there was a significant difference only between stressed and non-stressed seeds of the susceptible

cultivar. Moreover, the expression of *psbD* in this stage of maturation was higher in stressed seeds of the tolerant cultivar than in stressed seeds of the susceptible one.

Although we don't have quantification of these proteins, we hypothesize that in stressed seeds of the susceptible cultivar *psbA* and *psbD* are accumulated. The high Chl content in stressed seeds, especially in stressed seeds of the susceptible cultivar in R8 (Figure 9C and D), might be responsible for the higher expression of the core proteins of PSII, even late in maturation. He and Vermaas (1998) demonstrated that neither light nor Chl greatly modifies *psbA* transcript levels but that the availability of Chl is required for *psbA* translation. Also, the levels of phein a could be associated with the high expression of *psbA*. As described previously, phein a is an intermediate compound in the Chl breakdown pathway and it is also a component of the D1 protein (YAMATANI et al., 2013). There is the possibility that phein a is being accumulated in stressed seeds of the susceptible cultivar (as discussed previously), which could also explain higher expression or retention of D1 (*psbA*).

The retention of core subunits of PSII has been described in stay-green mutants that have impaired Chl degradation, which occurs also in stressed soybean seeds. The stay-green mutant non-yellow coloring 4 (*nyc4*) in rice (*Oryza sativa*) maintains high levels for the Fv/Fm value, which reflects PSII activity. In corroboration with that, degradation of the D1 and D2 core PSII subunits is nearly completely suppressed in the *nyc4* mutant. Thus, *NYC4* plays an important role in Chl degradation and PSII stability in the leaf senescence process (KUSABA et al., 2013). In addition, the PSII subunits CP43 (*psbC)* and CP47 (*psbB*) were also more stable in *nyc4-1* during senescence (YAMATANI et al., 2013).

The possible retention of the D1 (*psbA*) has to be further investigated since this protein has the highest turnover among all the PSII proteins, and this turnover can be affected by several conditions. Under optimal physiological conditions, the D1 protein already shows a light-dependent turnover which is several times higher than that of other chloroplast proteins (GIARDI et al., 1997). Under stressful conditions such as high luminosity the D1 subunit has an unusually high turnover rate that reflects the rapid replacement of photo-damaged D1 in PSII by newly synthesized D1 (NICKELSEN; RENGSTL, 2013). Taking this into account, the stresses applied in this study could, by itself, be the cause of higher expression of D1 (*psbA*) and D2 (*psbD*) in stressed seeds. Therefore it has to be highlighted here that the expression of these genes late in maturation

is dependent on the genotype. Under the same stressful conditions the changes in expression of *psbA* and *psbD* were different in seeds of the susceptible and tolerant cultivar, which means that not only a high turnover under stress would explain the higher expression of these genes in stressed soybean seeds.

As the core subunits of PSI also bind Chl, the expression of the genes (*psaA* and *psaB*) encoding for these proteins (A1 and A2), was also analyzed (Figure 30).

The expression of *psaA* and *psaB* displayed an increase from R6 to R7, followed by a decrease from R7 to R8. These genes were more highly expressed in stressed seeds of both cultivars in R6 and R7, but in R8 the expression was higher only in stressed seeds of the susceptible one. Yamatani et al. (2013) reported that *sgr* mutants in rice has a stronger effect on the stability of PSI-related proteins, including PSI subunits such as A1 (*psaA*) and A2 (*psaB*) and LHCI (*LHCA*) apoproteins, while *nyc4* has a major effect on PSII stability during leaf senescence. Thus, it is possible that A1 and A2 are also reteined in stressed/green soybean seeds due to the higher expression of psaA and psaB in these seeds.

Except for the expression of *psbA* that remains constant during maturation in stressed seeds of the susceptible cultivar, in general, the expression of the *LHCA*, *Cyt B6F* and the core proteins of PSI and PSII decreased in non-stressed seeds during maturation, and also decreased in stressed seeds albeit to a lower magnitude. This mostly reflects the normal disassembly of the photosynthetic apparatus during the maturation process that follows Chl degradation. As in stressed seeds Chl is retained at the end of the maturation, especially in seeds of the susceptible cultivar, it is expected that the proteins that binds Chl are also retained. In this study, the core proteins of both photosystems were the most affected. Basically, the expression of *psbA*, *psbD*, *psaA* and *psaB* was mostly higher in stressed seeds of the susceptible cultivar. However, to clearly understand which apoproteins are retained along with the Chl in stressed mature soybean seeds, the Chl binding proteins mentioned above should be quantified and in addition to that the LHCII proteins should be analyzed.

The permanence of Chl in leaves for longer period during crop senescence can be a desirable trait from an agronomical point of view. Functional stay-green mutations have been mentioned as a cause of advantageous effect on yield, which

was already attested in maize and rice stay-green mutants (THOMAS; HOWARTH, 2000; YOO et al., 2007). On the other hand, the presence of Chl in oilseeds has been associated with lower physiological quality as well as lower oil quality.

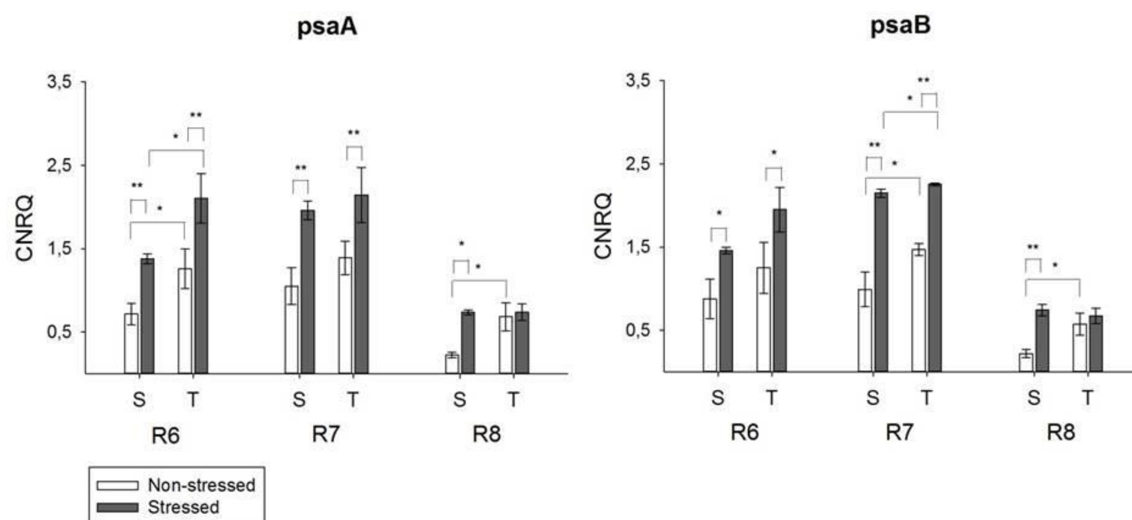


Figure 30. Gene expression analysis by real-time quantitative PCR (RT-qPCR) represented as the calibrated normalized relative quantity (CNRQ) for *psaA* and *psaB*. Gene expression is shown for non-stressed (open bars) and stressed seeds (closed bars) of the susceptible (S) and tolerant (T) cultivars in three stages of maturation (R6-R7-R8). Bars represent the mean values ± SE of three independent biological replicates. Bars connected by brackets represent the statistical comparisons. Asterisks represents statistically significant differences (*, P ≤ 0.05; **, P ≤ 0.01).

The negative effects of Chl presence on soybean seed quality was shown in this study and they have also been reported by Costa et al. (2001), Pádua et al.(2007) , Zorato et al. (2007), Pádua et al. (2009; 2009) and Cicero et al (2009). The same lower quality was mentioned for green seeds of *Brassica oleracea* (JALINK et al., 1998) and Arabidopsis green-seeded mutants (CLERKX et al., 2003; NAKAJIMA et al., 2012).

Two possibilities to explain the lower quality of the green seeds have been discussed. It is possible that the poor storability of the green seed phenotype have a causal relationship where the extra Chl may lead to oxidative damages (THOMAS;

HOWARTH, 2000; CLERKX et al., 2003). Otherwise, the presence of Chl is simply a marker of immaturity and hence lower quality of seeds for propagation (JALINK et al., 1998).

The presence of green color in mature seeds is an indication of impairments in the Chl degradation pathway and consequently in the degradation of the photosynthetic apparatus, both being, processes required for seed degreening during normal maturation.

A “mild” stay-green phenotype is displayed by soybean seeds of the susceptible cultivar when produced under combined heat and drought stresses. Such environmental conditions strictly affect the expression of genes encoding for some CCEs and core subunits of PSI and II. The expression of *NYC1_1*, *PPH2*, *PAO1* and *RCCR1* was significantly affected by the stress conditions, although it is the most important that they were differently impaired depending on the genotype. Likewise, the higher expression of *psbA*, *psbD*, *psaA* and *psaB* in stressed seeds of the susceptible cultivar is an indication that green seeds retain the core subunits of PSI and PSII during maturation.

Interestingly green soybean seeds are also impaired in the expression of two important genes *D1* and *D2*. The SGR protein encoded by these genes may be the regulator of the CCEs interaction with Chl-apoprotein complexes and consequently simultaneous proteolytic digestion of both Chl and apoproteins (SAKURABA et al., 2012). The significantly higher expression of *D1/D2* and *PHH2* in fully mature stressed seeds of the tolerant cultivar is probably the reason why even under a stressful environmental condition seeds of this genotype manage to degrade Chl to satisfactory levels.

Further investigation of enzyme activity, accumulation of intermediates of the Chl degradation pathway and also the retention of protein subunits of the photosystem should be considered. However, the gene expression results obtained in this study represent a breakthrough in understanding the soybean “green seed” problem. It will, as well, enable future studies on molecular markers that may help breeding programs to produce genetic materials more adapted to the hot and dry climate conditions of the main soybean production areas.

5. Conclusions

1. The effect of heat and drought stress on green soybean seed production is dependent on the genotype.

2. The presence of chlorophyll in mature soybean seeds is an indication of lower seed quality.

3. The chlorophyll degradation pathway is impaired in maturing soybean seeds produced under heat and drought stress.

4. Multiple ontologies were enriched in soybean seeds produced under stress. Among the top-ranking was the photosynthetic process, particularly the photosystem (PS) II category. Expression analysis suggests higher levels of the proteins D1 (*psbA*), D2 (*psbD*), A1 (*psaA*) and A2 (*psaB*) core subunits of PSI and PSII, in stressed/green seeds.

5. *PPH2*, *D1* and *D2* are the most affected genes in stressed/green seeds of the susceptible cultivar and may be the major cause of the chlorophyll retention in soybean seeds.

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