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Author
Pater, Dianne

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Examining natural variation in drought responses in *Brassica napus*

A dissertation submitted in partial satisfaction

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in

Biology

by

Dianne T. Pater

Committee in charge:

Professor Julian I. Schroeder, Chair
Professor Nigel Crawford
Professor James Golden
Professor Ralph Keeling
Professor Yunde Zhao

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The dissertation of Dianne T. Pater is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

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DEDICATION

I dedicate this dissertation to my family and friends who have provided me with support over the years. Thank you for your certainty that I could reach this point and for helping me get there.

To Dave Hanson for taking that first chance on me as a scientist.

To Greg for all the years, love, and support.

And to Cheyenne and Sierra: you are my heart and my joy and my inspiration for everything.

EPIGRAPH

A plant that lives where it should not is simply a pest, but a plant that thrives where it should not live is a weed. We don't resent the audacity of the weed, as every seed is audacious; we resent its fantastic success.

Hope Jahren

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LIST OF ABBREVIATIONS

A	photosynthetic CO_2 assimilation rate
g_s	stomatal conductance
J_{max}	maximum electron transport rate
PAR	photosynthetically active radiation
ppm	parts per million
‰	per thousand
RuBP	ribulose-1,5-bisphosphate
TE	transpiration efficiency
V_{cmax}	maximum Rubisco carboxylation rate
WUE	water use efficiency

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Chapter 1

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VITA

2011 Bachelor of Science, Biology, *summa cum laude*
University of New Mexico

2017 Doctor of Philosophy, Biology
University of California, San Diego

PUBLICATIONS

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Sutimantanapi, D., Pater, D. and Smith, L. G. (2014) 'Divergent roles for maize PAN1 and PAN2 receptor-like proteins in cytokinesis and cell morphogenesis.', *Plant physiology*, 164(4), pp. 1905–17.

Heckwolf, M., Pater, D., Hanson, D. T., and Kaldenhoff, R. (2011) 'The Arabidopsis thaliana aquaporin AtPIP1 ; 2 is a physiologically relevant CO 2 transport facilitator', *The Plant Journal*, 67(5), pp. 795–804.

ABSTRACT OF THE DISSERTATION

Examining natural variation in drought responses in *Brassica napus*

by

Dianne T. Pater

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Professor Julian I. Schroeder, Chair

Drought is a major stress which reduces crop yields, and which will continue to be an increasing problem in the coming years as climate change and limited fresh water supplies lead to higher temperatures, desertification, and increased soil salinity. These environmental stresses can significantly impact both the seed yield and quality of crops. There are several strategies which plants utilize to mitigate the effects of water deficit, making the identification of specific traits which convey drought tolerance difficult. As

drought tolerance is a complex trait, accurate phenotyping to select for resilient genotypes is needed to improve our understanding of plant drought responses.

In this study, stable carbon isotope screening ($\delta^{13}C$) of a diversity set of the crop plant *Brassica napus* grown in the field was used to identify accessions with traits linked with extremes in water use efficiency (WUE). We investigated physiological characteristics of the selected accessions to identify how these characteristics translate to differences in WUE. Using gas exchange techniques, we identified an interesting spring-type accession (G302, Mozart), which exhibited the highest WUE in the field, based on $\delta^{13}C$ measurements. This line displayed high CO_2 assimilation rates coupled with an increased electron transport capacity (J_{max}) under lab conditions. We also analyzed stomatal conductance response to exogenous abscisic acid (ABA) in the selected accessions. While little variation was observed in the response rates of spring-type accessions, one semi-winter accession demonstrated a significantly more rapid response to exogenous ABA, which was in line with a higher WUE derived from $\delta^{13}C$ measurements. This research supports the genetic data showing distinct genetic lineages for spring and semi-winter accessions. It also illustrates the importance of examining natural variation at a physiological level for understanding the underlying mechanisms of drought responses.

Introduction

The Green Revolution focused on increasing crop productivity by optimizing growing environments. This was accomplished both through breeding for growth-linked yield traits (dwarf wheats and rice, etc.) and through agronomic practices such as irrigation, fertilizing suboptimal soils, and the use of pesticides and herbicides. Plant breeders and researchers concurrently developed high yielding crops which thrived under those improved conditions. With increasing world population and changing water consumption due to improving global living standards, the global demand for water is threatening sustainable development. To meet the growing food and fuel needs of the global population, plant breeders and researchers will need to maintain or improve on the work started in the green revolution, in order to feed the additional population and to counteract effects of climate change on crop production.

A number of environmental stresses adversely affect plant growth and impact the distribution of species. Some examples of abiotic stresses plants may encounter include extremes of temperature, excess light, soil salinity, and drought. It is estimated that two-thirds of the global population live under water scarcity conditions at least one month of the year (Mekonnen and Hoekstra, 2016). Crop productivity can be severely impacted by exposure to an unfavorable environment, with more than half of the maximum potential yield lost under stressful conditions (Boyer, 1982). This reduction in crop yield can lead to devastating losses both economically and in terms of food security (Battisti and Naylor, 2009). As stated by Bill Gates at the International Fund for Agricultural Development Governing Council meeting in 2012, “Investments in

agriculture are the best weapons against hunger and poverty." With anticipated increases in both global population and climate change effects on water availability, improving stress tolerance in plants has become an important focus of research.

Drought effects on crops

Inadequate water supplies are one of the most important limitations to plant health and crop yield. Freshwater scarcity has been listed by the World Economic Forum as the largest global risk in terms of potential impact on economies, environments, and people (World Economic Forum, 2015). Nearly 70% of the world's fresh water consumption is used in agricultural activities (WWAP - United Nations World Water Assessment Programme, 2015). Global climate change affects not only water availability from rainfall but also increases temperature and evapotranspiration due to the higher temperature (Trenberth *et al.*, 2014; Gray *et al.*, 2016). As a result of the anticipated shortage of water for agricultural use, the International Water Management Institute called for a 40% improvement by 2025 in yields of crops where water is the limiting factor (IWMI, 2013). Improvement of crop productivity during periodic and/or sustained periods of drought is a fundamental challenge for the agricultural industry.

Drought can be defined as a, "period of abnormally dry weather sufficiently prolonged for the lack of precipitation to cause a serious hydrological imbalance," (Trenberth *et al.*, 2014). In the context of agriculture, however, drought describes conditions where the available water is less than that required by the plant to sustain maximum growth and productivity (Boyer, 1982; Deikman, Petracek and Heard, 2012).

The defining component of drought is the decrease in availability of soil water, which can be quantified as a decrease in soil water potential (Kramer and Boyer, 1995). Terminal drought refers to a progressive decrease in available water which ultimately results in plant death. Intermittent drought is the result of episodes of insufficient water during the growth season which may or may not result in plant death (Neumann, 2008).

A variety of strategies are employed by plants to respond to drought stress. Some species of plants are able to tolerate reduced water content via mechanisms to avoid cellular damage (Verslues *et al.*, 2006). Desiccation-tolerant plants can survive a fully dried state by entering a metabolically dormant state similar to seed dormancy (Oliver, Cushman and Koster, 2010). Most crop plants, however are mesophytes, which are adapted to environments that are not extremely wet or dry. As such, crop plants cannot enter a dormant state and are unable to recover from a severe decrease (roughly 50% loss) in water content (Verslues *et al.*, 2006). Some plants escape abiotic stress by accelerating flowering to ensure reproduction before the onset of severe drought (Neumann, 2008). This “drought escape” survival strategy is a possible goal for crop plants that are able to achieve the desired biomass prior to terminal drought.

A more common strategy in crop plants is stress and dehydration avoidance. Plants can avoid dehydration by accumulation of solutes to prevent water loss and adjust osmotic potential (Kramer and Boyer, 1995). Plants can also alter physiological features to increase water uptake and/or reduce water loss. Access to water in the soil is improved through efficient root architecture. Root traits associated with improved drought avoidance include small fine root diameters, deep roots, and root length

density (Passioura, 1983; Pinheiro *et al.*, 2005; Comas *et al.*, 2013). Plants can reduce water loss to the environment by decreasing leaf cuticle permeability (Goodwin and Jenks, 2007) and by closing stomata in the leaf epidermis (Davies, Wilkinson and Loveys, 2002).

As there are many strategies which plants utilize to mitigate the effects of water deficit, identifying specific traits which convey drought tolerance has proven to be difficult. Drought stress and its effects are perceived throughout the plant, including changes in gene expression and physiological processes (Kasuga *et al.*, 1999; Shinozaki, Yamaguchi-Shinozaki and Seki, 2003; Pinheiro and Chaves, 2011). Confounding the issue further is that the goal of breeding for drought tolerance is not merely survival, but maintenance of yield. Since drought tolerance is a complex trait with many indicators, accurate, high-throughput phenotyping to select for resilient genotypes is needed for researchers and plant breeders alike.

Drought stress elicits complex whole-plant physiological and morphological responses. When plants perceive water deficit, the phytohormone abscisic acid (ABA) is synthesized in and/or transported to leaf tissue. The increased concentration of ABA triggers a cascade of responses that promote stomatal closure via loss of turgor of the specialized guard cells which form the stomatal opening (Schroeder *et al.*, 2001; Schroeder, Kwak and Allen, 2001; Hauser, Waadt and Schroeder, 2011). Accumulation of ABA also inhibits stomatal opening to preserve plant hydration (De Silva, Hetherington and Mansfield, 1985; Schroeder *et al.*, 2001).

Water Use Efficiency

The term water-use efficiency (WUE) has been used to describe different scales of observations from whole plant, crop, or leaf level, and including instantaneous and whole-life timescales. At the crop level, WUE can be defined as a ratio of biomass accumulation to water consumed. The biomass accumulation can be expressed as photosynthetic carbon assimilation, total crop biomass, or crop grain yield; while water consumed can represent transpiration (loss of water by evaporation from terrestrial plants), evapotranspiration (which is the sum of plant transpiration and evaporation from land and ocean surfaces), or total water input to the system (Sinclair, Tanner and Bennett, 1984). It can also represent a wide range of timescales from days, months, a growing season, or a year (Morison *et al.*, 2008). At the whole plant level, transpiration efficiency (TE) is defined as the ratio of biomass:water transpired (Vadez *et al.*, 2014). Measuring plant level TE directly is problematic as it involves assessment of biomass increases and plant water usage on a long-term basis.

Perhaps the most widely used measurement to examine water productivity is intrinsic transpiration efficiency, or 'instantaneous WUE,' which is the ratio of instantaneous CO_2 assimilation (*A*) to transpiration (*E*) at the stomata (Hsiao, Steduto and Fereres, 2007; Vadez *et al.*, 2014). These factors are closely tied to the concentration gradient of either CO_2 ($c_a - c_i$) or water vapor ($w_i - w_a$) between the air outside the leaf and the air within the leaf. As shown in Condon et al., 2002,

$$TE \approx 0.6\, c_a(1 - c_i/c_a)/(w_i - w_a) \qquad (1)$$

where the factor 0.6 refers to the relative diffusivities of CO_2 and water vapor in air, c_i and c_a are the CO_2 concentrations within the leaf and ambient air, respectively, and w_i and w_a are the stomatal and ambient vapor pressures. Based on this equation, improvements in TE can theoretically be achieved either through lowered stomatal conductance or higher photosynthetic capacity, or a combination of both (Condon *et al.*, 2002). It is important to recognize, however, that a reduction in stomatal conductance can lead to concurrent reductions ci/ca, which may translate to a reduction in assimilation (*A*). Because transpiration can vary with air humidity and leaf temperature, some researchers instead focus on the relationship between assimilation (*A*) and stomatal conductance (g_s), which is sometimes referred to as, 'intrinsic WUE' (Morison *et al.*, 2008). The ratio of A/g_s is linearly related to the intercellular partial pressure of CO_2 (c_i) (Morison *et al.*, 2008)

Carbon Isotope Discrimination

The majority of carbon on Earth is ^{12}C (98.9%) with the heavy stable isotope ^{13}C comprising approximately 1.1% of carbon globally. The isotopes occur in uneven ratios within different compounds, which can provide information about biological and carbon cycle processes including carbon fixation, respiration and food chains (Nier and Gulbransen, 1939; Park and Epstein, 1960; O'Leary, 1981; van der Merwe, 1982). It was discovered that the photosynthetic enzyme Rubisco discriminates against ^{13}C (Park and Epstein, 1960) due to the lower reactivity of ^{13}C (Melander and Saunders, 1980; Hermes *et al.*, 1982). Based on the isotopic composition of the air, which is approximately - 8‰

with respect to the Pee Dee belemnite standard (Keeling, Mook and Tans, 1979), and the enzymatic discrimination by Rubisco (Whelan and Sackett, 1973; Christeller, Laing and Troughton, 1976; Wong, Benedict and Kohel, 1979), it was determined that enzymatic fractionation alone did not account for $\delta^{13}C$ values found in vivo (O'Leary, 1981; Farquhar, O'Leary and Berry, 1982).

A new model showed CO_2 diffusion, metabolism, and decarboxylation processes can significantly affect carbon isotope discrimination (Farquhar, O'Leary and Berry, 1982; Farquhar and Richards, 1984). Researchers also noted the ^{13}C /^{12}C ratio ($\delta 1^{13}C$) varied with different CO_2 fixation pathways. Plants with the C3 pathway of carbon assimilation have an average $\delta^{13}C$ approximately 10‰ less than that of plants with the dicarboxylic acid (C4) pathway (Bender, 1971; Whelan and Sackett, 1973). Additionally, plants utilizing crassulacean acid metabolism (CAM) display intermediate values (Bender, 1971), which have been attributed to the use of both C3 and C4 metabolism in these species (Osmond *et al.*, 1973). As C4 and CAM species have higher WUE, and are more enriched in ^{13}C than C3 species, it was hypothesized that $\delta^{13}C$ values could be used as a comparative measure of WUE (Farquhar, O'Leary and Berry, 1982).

Isotopic analyses of wheat plants, for which WUE was determined using long-term accumulation of biomass and water use, demonstrated the relationship between carbon isotope discrimination ($\Delta^{13}C$) and WUE (Farquhar and Richards, 1984). It was demonstrated that $\Delta^{13}C$ is positively related to c_i/c_a, and thus negatively related to WUE. This negative relationship has been demonstrated in several crop species, including wheat (Condon, Richards and Farquhar, 1987; Knight, Livingston and van Kessel, 1994;

Sayre, Acevedo and Austin, 1995), barley (Hubick and Farquhar, 1989; Craufurd *et al.*, 1991), common bean (*Phaseolus vulgaris* L.) (Ehleringer, 1990; Donovan and Ehleringer, 1994), and canola (Knight, Livingston and van Kessel, 1994). The relationship between WUE and $\Delta^{13}C$ has also been shown to be not only genetically controlled, but also subject to change with varied watering regimes (Ismail and Hall, 1993; Knight, Livingston and van Kessel, 1994; Monneveux *et al.*, 2006). A major drawback of this method, however, is that it does not distinguish whether differences in $\Delta^{13}C$ are driven by photosynthetic efficiency or improved conductance.

Genetic variation

Agricultural practices have relied upon the domestication of wild plant species with desirable traits. Over thousands of years, farmers and breeders have cultivated and selected plants based on traits such as nutritional value, stress adaptation, and yield. Domestication leads to rapid enrichment for certain traits, while subsequently reducing the frequency of undesirable traits. Genetic analysis of modern maize (*Zea mays* ssp. *mays*) as compared to early domesticated maize, and wild teosinte grass (*Zea mays* ssp. *parviglumis*) identified nearly 1200 genes throughout the genome have been affected by artificial selection (Wright *et al.*, 2005).

Crop varieties have been selected for adaptation to local conditions. As a result, a wealth of genetic diversity exists in land races and “folk” varieties of many crops which may not be available in widely grown domesticated varieties (Cleveland, Soleri and Smith, 1994). The process of breeding has created crops with reduced genetic diversity.

Resistance to both biotic and abiotic stresses can be higher in heirloom varieties, possibly as a result of diversity in resistance genes as compared to a reduced variety of resistance genes in modern varieties (Tanksley and McCouch, 1997). Considerable interspecies genetic variation has been shown in WUE (Farquhar and Richards, 1984). By exploiting the genetic material from land races, folk varieties, and wild relatives, breeders may identify genes involved in useful agronomic traits (Tanksley and McCouch, 1997; Rieseberg, Baird and Gardner, 2000; Quist and Chapela, 2001; Stewart, Halfhill and Warwick, 2003).

Brassica napus

The plant family *Brassicaceae* is widely distributed globally and includes over 3000 species. Amongst its important species are the model research species *Arabidopsis thaliana* and several crops, including many vegetable crops (broccoli, cauliflower, cabbage, kale) and oilseed crops (rapeseed). Brassica species are grown for both food oil and for biofuel stocks. *Brassica napus* is an amphidiploid species, arising from the hybridization between the diploid species *B. rapa* and *B. oleracea* approximately 10,000 years ago (Parkin *et al.*, 2005). *B. napus* is of particular interest both for its agronomic importance as well as its genomic similarity to *Arabidopsis*.

B. napus is an economically important oilseed crop with over 36 million hectares grown worldwide for a global yield of nearly 74 million metric tons (Food and Agriculture Organization of the United Nations (FAO, 2014). The nutritional fatty acid content of rapeseed oil makes it an attractive food oil, and is currently the third largest

source of global vegetable oil (Food and Agriculture Organization of the United Nations (FAO), 2014). Additionally, the rapeseed meal remaining after oil processing serves as a high protein food source for livestock and aquaculture, representing 7% of vegetable meal consumed by European livestock (FEDIOL, 2007). Rapeseed oil also represents an important source of renewable fuel, producing greater than 65% of EU biodiesel (FEDIOL, 2007).

Water deficit can affect *B. napus* during all stages of growth, influencing processes such as photosynthesis, protein synthesis, and metabolite accumulation. These processes can affect seed yield and quality, either directly or indirectly (Jensen, Mogensen, Mortensen, Andersen, *et al.*, 1996; Hashem *et al.*, 1998; Sangtarash *et al.*, 2009). As in most terrestrial plants, ABA production increases in *B. napus* upon perception of drought stress (Qaderi, Kurepin and Reid, 2006). Stressed plants also exhibit decreased net CO_2 assimilation, chlorophyll content and transpiration (Hashem *et al.*, 1998; Din *et al.*, 2011; Qaderi, Kurepin and Reid, 2012; Shafiq *et al.*, 2014)

Water shortage at any stage of development can have potentially damaging effects on seed quality and yield in *B. napus.* Drought stress has a severe impact on yield in *B. napus*, resulting in reduced pod number, seed number, and seed weight, with a reduction in seed yield of 20-40% observed in stressed vs. nonstressed plants (Ahmadi and Bahrani, 2009). Water deficit conditions can also decrease seed oil content (Bouchereau *et al.*, 1996; Champolivier and Merrien, 1996; Moaveni, Ebrahimi and Farahani, 2010) and alter seed composition (Enjalbert *et al.*, 2013).

Drought tolerance is a complex, multi-genic trait, which complicates crop improvement. Mechanisms and genes involved in conferring drought tolerance may be identified by exploring natural variations between accessions of a species (Donovan and Ehleringer, 1994; Barbour *et al.*, 2010). Breeders can utilize the inter- and intraspecific diversity found in wild relatives as well as diverse domestic accessions to increase genetic variability within crops. Our understanding of the physiological basis of WUE in *B. napus* can be improved by examining differences between diverse accessions (Zhu *et al.*, 2016).

In this dissertation, we present work that explores drought adaptation in the crop plant, *Brassica napus*. Chapter 1 of the thesis focuses on a diversity set of *B. napus* to dissect the mechanisms involved in naturally occurring variations of WUE in the field, as identified using $\delta^{13}C$ measurements. A variety of approaches were utilized, including gas exchange to measure photosynthetic efficiency and ABA responsiveness. Appendix 1 presents a review of molecular and systems approaches to inform strategies for improvement of drought tolerance in *B. napus.* Appendix 2 presents a project using RNAi to determine the effect of PP2C down-regulation in guard cells and in response to drought in whole plants. Appendix 3 presents experiments that tested new protocols for simulating drought conditions and using thermal imaging to assay plant responses to decreasing soil moisture.

REFERENCES

Ahmadi, M. and Bahrani, M. J. (2009) 'Yield and yield components of rapeseed as influenced by water stress at different growth stages and nitrogen levels', American-Eurasian Journal of Agricultural and Environmental Science, 5(6), pp. 755–761.

Barbour, M. M., Warren, C. R., Farquhar, G. D., Forrester, G. and Brown, H. (2010) 'Variability in mesophyll conductance between barley genotypes, and effects on transpiration efficiency and carbon isotope discrimination.', Plant, Cell & Environment, 33(7), pp. 1176–85. doi: 10.1111/j.1365-3040.2010.02138.x.

Battisti, D. S. and Naylor, R. L. (2009) 'Historical warnings of future food insecurity with unprecedented seasonal heat', Science, 323(5911), pp. 240–244. doi: 10.1126/science.1164363.

Bender, M. M. (1971) 'Variations in the 13C/12C ratios of plants in relation to the pathway of photosynthetic carbon dioxide fixation', Phytochemistry, 10(6), pp. 1239–1244. doi: 10.1016/S0031-9422(00)84324-1.

Bouchereau, A., Clossais-Besnard, N., Bensaoud, A., Leport, L. and Renard, M. (1996) 'Water stress effects on rapeseed quality', European Journal of Agronomy, 5, pp. 19–30.

Boyer, J. S. (1982) 'Plant productivity and environment.', Science, 218(4571), pp. 443–448. doi: 10.1126/science.218.4571.443.

Champolivier, L. and Merrien, A. (1996) 'Effects of water stress applied at different growth stages to Brassica napus L. var. oleifera on yield, yield components and seed quality', 5, pp. 153–160.

Christeller, J. T., Laing, W. A. and Troughton, J. H. (1976) 'Isotope discrimination by Ribulose 1, 5-Diphosphate Carboxylase', Plant Physiology, 57, pp. 580–582.

Cleveland, D. A., Soleri, D. and Smith, S. E. (1994) 'Do folk crop varieties have a role in sustainable agriculture?', BioScience, 44(11), pp. 740–751. doi: 10.2307/1312583.

Comas, L. H., Becker, S. R., Cruz, V. M. V., Byrne, P. F. and Dierig, D. A. (2013) 'Root traits contributing to plant productivity under drought', Frontiers in Plant Science, 4(November), pp. 1–16. doi: 10.3389/fpls.2013.00442.

Condon, A. G., Richards, R. A. and Farquhar, G. D. (1987) 'Carbon isotope discrimination is positively correlated with grain yield and dry matter production in field-grown

wheat', Crop Science. Crop Science Society of America, 27(5), p. 996. doi: 10.2135/cropsci1987.0011183X002700050035x.

Condon, A. G., Richards, R. A., Rebetzke, G. J. and Farquhar, G. D. (2002) 'Improving intrinsic water use efficiency and crop yield', Crop Science, 42, pp. 122–131. doi: 10.2135/cropsci2002.0122.

Craufurd, P. Q., Austin, R. B., Acevedo, E. and Hall, M. A. (1991) 'Carbon isotope discrimination and grain-yield in barley', Field Crops Research, 27, pp. 301–313.

Davies, W. J., Wilkinson, S. and Loveys, B. (2002) 'Stomatal control by chemical signalling and the exploitation of this mechanism to increase water use efficiency in agriculture', New Phytologist, 153(3), pp. 449–460. doi: 10.1046/j.0028-646X.2001.00345.x.

Deikman, J., Petracek, M. and Heard, J. E. (2012) 'Drought tolerance through biotechnology: Improving translation from the laboratory to farmers' fields', Current Opinion in Biotechnology. Elsevier Ltd, 23(2), pp. 243–250. doi: 10.1016/j.copbio.2011.11.003.

Din, J., Khan, S. U., Ali, I. and Gurmani, A. R. (2011) 'Physiological and agronomic response of canola varieties to drought stress', The Journal of Animal and Plant Sciences, 21(1), pp. 78–82.

Donovan, L. A. and Ehleringer, J. R. (1994) 'Carbon isotope discrimination, water-use efficiency, growth, and mortality in a natural shrub population', Oecologia, 100(3), pp. 347–354. doi: 10.1007/bf00316964.

Ehleringer, J. R. (1990) 'Correlations between carbon isotope discrimination and leaf conductance to water-vapor in common beans', Plant Physiology, 93(4), pp. 1422–1425. doi: 10.1104/pp.93.4.1422.

Enjalbert, J. N., Zheng, S., Johnson, J. J., Mullen, J. L., Byrne, P. F. and McKay, J. K. (2013) 'Brassicaceae germplasm diversity for agronomic and seed quality traits under drought stress', Industrial Crops and Products. Elsevier B.V., 47, pp. 176–185. doi: 10.1016/j.indcrop.2013.02.037.

Farquhar, G. D. and Richards, R. A. (1984) 'Isotopic composition of plant carbon correlates with water-use efficiency of wheat genotypes', Australian Journal of Plant Physiology, 11(1), pp. 539–552.

Farquhar, G., O'Leary, M. and Berry, J. (1982) 'On the relationship between carbon isotope discrimination and the intercellular carbon dioxide concentration in

leaves', Australian Journal of Plant Physiology, 9(2), p. 121. doi: 10.1071/PP9820121.

FEDIOL (2007) Food, Feed and Fuels: A Snapshot.

Food and Agriculture Organization of the United Nations (FAO) (2014) FAO Statistical Yearbook 2014- Asia and the Pacific Food and Agriculture. doi: http://www.fao.org/docrep/018/i3107e/i3107e.PDF.

Goodwin, S. M. and Jenks, M. A. (2007) 'Plant cuticle function as a barrier to water loss', in Plant Abiotic Stress, pp. 14–31. doi: 10.1002/9780470988503.ch1.

Gray, S. B., Dermody, O., Klein, S. P., Locke, A. M., McGrath, J. M., Paul, R. E., Rosenthal, D. M., Ruiz-Vera, U. M., Siebers, M. H., Strellner, R., Ainsworth, E. A., Bernacchi, C. J., Long, S. P., Ort, D. R. and Leakey, A. D. B. (2016) 'Intensifying drought eliminates the expected benefits of elevated carbon dioxide for soybean', Nature Plants. Nature Publishing Group, 2(9), p. 16132. doi: 10.1038/nplants.2016.132.

Hashem, A., Majumdar, A., Hamid, A. and Hossain, M. M. (1998) 'Drought stress effects on seed yield, yield attributes, growth, cell membrane stability and gas exchange of synthesized Brassica napus L.', Journal of Agronomy and Crop Science, 136, pp. 129–136.

Hauser, F., Waadt, R. and Schroeder, J. I. (2011) 'Evolution of abscisic acid synthesis and signaling mechanisms.', Current Biology. Elsevier Ltd, 21(9), pp. R346-55. doi: 10.1016/j.cub.2011.03.015.

Hermes, J. D., Roeske, C. A., O'Leary, M. H. and Cleland, W. W. (1982) 'Use of multiple isotope effects to determine enzyme mechanisms and intrinsic isotope effects. Malic Enzyme and Glucose-6-phosphate Dehydrogenase', Biochemistry, 21(20), pp. 5106–5114. doi: 10.1021/bi00263a040.

Hsiao, T. C., Steduto, P. and Fereres, E. (2007) 'A systematic and quantitative approach to improve water use efficiency in agriculture', Irrigation Science, 25(3), pp. 209–231. doi: 10.1007/s00271-007-0063-2.

Hubick, K. T. and Farquhar, G. D. (1989) 'Carbon isotope discrimination and the ratio of carbon gained to water lost in barley cultivars', Plant, Cell and Environment, 12, pp. 795–804.

Ismail, A. M. and Hall, A. E. (1993) 'Carbon isotope discrimination and gas exchange of cowpea accessions and hybrids', Crop Science, 33(4), pp. 788–793. doi: 10.2135/cropsci1993.0011183X003300040032x.

IWMI (2013) Annual report 2013. doi: 10.1111/epp.12066.

Jensen, C. R., Mogensen, V. O., Mortensen, G., Andersen, M. N., Schjoerring, J. K., Thage, J. H. and Koribidis, J. (1996) 'Leaf photosynthesis and drought adaptation in field-grown oilseed rape (Brassica napus L.)', Australian Journal of Plant Physiology, 23, pp. 631–644.

Kasuga, M., Liu, Q., Miura, S., Yamaguchi-Shinozaki, K. and Shinozaki, K. (1999) 'Improving plant drought, salt and freezing tolerance by gene transfer of a single stress-inducible transcription factor.', Nature Biotechnology, 17, pp. 287–291. doi: 10.1038/7036.

Keeling, C. D., Mook, W. G. and Tans, P. P. (1979) 'Recent trends in the 13C/12C ratio of atmospheric carbon dioxide', Nature, pp. 121–123. doi: 10.1038/277121a0.

Knight, J. D., Livingston, N. J. and van Kessel, C. (1994) 'Carbon isotope discrimination and water-use efficiency of six crops grown under wet and dryland conditions', Plant, Cell & Environment, 17(2), pp. 173–179. doi: 10.1111/j.1365-3040.1994.tb00280.x.

Kramer, P. J. and Boyer, J. S. (1995) Water Relations of Plants and Soils, Academic Press. doi: 10.1097/00010694-199604000-00007.

Mekonnen, M. M. and Hoekstra, A. Y. (2016) 'Four billion people facing severe water scarcity', Science Advances, 2(2), pp. e1500323–e1500323. doi: 10.1126/sciadv.1500323.

Melander, L. C. S. and Saunders, W. H. (1980) 'Reaction rates of isotopic molecules', John Wiley & Sons.

van der Merwe, N. J. (1982) 'Carbon Isotopes, Photosynthesis, and Archaeology: Different pathways of photosynthesis cause characteristic changes in carbon isotope ratios that make possible the study of prehistoric human diets', American Scientist, 70(6), pp. 596–606.

Moaveni, P., Ebrahimi, A. and Farahani, H. A. (2010) 'Studying of oil yield variations in winter rapeseed (Brassica napus L.) cultivars under drought stress conditions', 2(May), pp. 71–75.

Monneveux, P., Rekika, D., Acevedo, E. and Merah, O. (2006) 'Effect of drought on leaf gas exchange, carbon isotope discrimination, transpiration efficiency and productivity in field grown durum wheat genotypes', Plant Science, 170, pp. 867–872. doi: 10.1016/j.plantsci.2005.12.008.

Morison, J. I., Baker, N., Mullineaux, P. and Davies, W. (2008) 'Improving water use in crop production', Philosophical Transactions of the Royal Society B: Biological Sciences, 363(1491), pp. 639–658. doi: 10.1098/rstb.2007.2175.

Neumann, P. M. (2008) 'Coping mechanisms for crop plants in drought-prone environments', Annals of Botany, 101(7), pp. 901–907. doi: 10.1093/aob/mcn018.

Nier, A. O. and Gulbransen, E. (1939) 'Variations in the relative abundance of the carbon isotopes', Journal of the American Chemical Society, 61, pp. 697–698. doi: 10.1038/1691051a0.

O'Leary, M. H. (1981) 'Carbon isotope fractionation in plants', Phytochemistry, 20(4), pp. 553–567. doi: 10.1016/0031-9422(81)85134-5.

Oliver, M. J., Cushman, J. C. and Koster, K. L. (2010) Dehydration tolerance in plants, Plant Stress Tolerance. Edited by R. Sunkar. Totowa, NJ: Humana Press (Methods in Molecular Biology). doi: 10.1007/978-1-60761-702-0.

Osmond, C. B., Allaway, W. G., Sutton, B. G., Troughton, J. H., Queiroz, O., Luttge, U. and Winter, K. (1973) 'Carbon isotope discrimination in photosynthesis of crassulacean-acid metabolism plants', Nature, 246(5427), pp. 41–42. doi: 10.1038/246041a0.

Park, R. and Epstein, S. (1960) 'Carbon isotope fractionation during photosynthesis', Geochimica et Cosmochimica Acta, 21(1958), pp. 110–126.

Parkin, I. A. P., Gulden, S. M., Sharpe, A. G., Lukens, L., Trick, M., Osborn, T. C. and Lydiate, D. J. (2005) 'Segmental structure of the Brassica napus genome based on comparative analysis with Arabidopsis thaliana.', Genetics, 171(2), pp. 765–81. doi: 10.1534/genetics.105.042093.

Passioura, J. B. (1983) 'Roots and drought resistance', Agricultural Water Management, 7(1–3), pp. 265–280. doi: 10.1016/0378-3774(83)90089-6.

Pinheiro, C. and Chaves, M. M. (2011) 'Photosynthesis and drought: Can we make metabolic connections from available data?', Journal of Experimental Botany, 62(3), pp. 869–882. doi: 10.1093/jxb/erq340.

Pinheiro, H. A., DaMatta, F. M., Chaves, A. R. M., Loureiro, M. E. and Ducatti, C. (2005) 'Drought tolerance is associated with rooting depth and stomatal control of water use in clones of Coffea canephora', Annals of Botany, 96(1), pp. 101–108. doi: 10.1093/aob/mci154.

Qaderi, M. M., Kurepin, L. V. and Reid, D. M. (2006) 'Growth and physiological responses of canola (Brassica napus) to three components of global climate change: Temperature, carbon dioxide and drought', Physiologia Plantarum, 128, pp. 710–721. doi: 10.1111/j.1399-3054.2006.00804.x.

Qaderi, M. M., Kurepin, L. V. and Reid, D. M. (2012) 'Effects of temperature and watering regime on growth, gas exchange and abscisic acid content of canola (Brassica napus) seedlings', Environmental and Experimental Botany. Elsevier B.V., 75, pp. 107–113. doi: 10.1016/j.envexpbot.2011.09.003.

Quist, D. and Chapela, I. H. (2001) 'Transgenic DNA introgressed into traditional maize landraces in Oaxaca, Mexico', Nature, 414(6863), pp. 541–543. doi: 10.1038/35107068.

Rieseberg, L. H., Baird, S. J. E. and Gardner, K. A. (2000) 'Hybridization, introgression, and linkage evolution', Plant Molecular Biology, 42(1), pp. 205–224. doi: 10.1023/A:1006340407546.

Sangtarash, M. H., Qaderi, M. M., Chinnappa, C. C. and Reid, D. M. (2009) 'Differential sensitivity of canola (Brassica napus) seedlings to ultraviolet-B radiation, water stress and abscisic acid', Environmental and Experimental Botany, 66(2), pp. 212–219. doi: 10.1016/j.envexpbot.2009.03.004.

Sayre, K. D., Acevedo, E. and Austin, R. B. (1995) 'Carbon isotope discrimination and grain yield for three bread wheat germplasm groups grown at different levels of water stress', Field Crops Research, 41, pp. 45–54.

Schroeder, J. I., Allen, G. J., Hugouvieux, V., Kwak, J. M. and Waner, D. (2001) 'Guard cell signal transduction', Annual Review of Plant Physiology and Plant Molecular Biology, 52(1), pp. 627–658. doi: 10.1146/annurev.arplant.52.1.627.

Schroeder, J. I., Kwak, J. M. and Allen, G. J. (2001) 'Guard cell abscisic acid signalling and engineering drought hardiness in plants.', Nature, 410(6826), pp. 327–330. doi: 10.1038/35066500.

Shafiq, S., Akram, N. A., Ashraf, M. and Arshad, A. (2014) 'Synergistic effects of drought and ascorbic acid on growth, mineral nutrients and oxidative defense system in canola (Brassica napus L.) plants', Acta Physiologiae Plantarum, pp. 1539–1553. doi: 10.1007/s11738-014-1530-z.

Shinozaki, K., Yamaguchi-Shinozaki, K. and Seki, M. (2003) 'Regulatory network of gene expression in the drought and cold stress responses', Current Opinion in Plant Biology, 6(5), pp. 410–417. doi: 10.1016/S1369-5266(03)00092-X.

De Silva, D. L. R., Hetherington, A. M. and Mansfield, T. A. (1985) 'Synergism between calcium ions and abscisic acid in preventing stomatal opening', New Phytologist, 100, pp. 473–482.

Sinclair, T. R., Tanner, C. B. and Bennett, J. M. (1984) 'Water-Use Efficiency in Crop Production', BioScience, 34(1), pp. 36–40. doi: 10.2307/1309424.

Stewart, C. N., Halfhill, M. D. and Warwick, S. I. (2003) 'Genetic modification: Transgene introgression from genetically modified crops to their wild relatives', Nature Reviews Genetics, 4(10), pp. 806–817. doi: 10.1038/nrg1179.

Tanksley, S. D. and McCouch, S. R. (1997) 'Seed Banks and Molecular Maps: Unlocking Genetic Potential from the Wild', Science, 277(5329), pp. 1063–1066. doi: 10.1126/science.277.5329.1063.

Trenberth, K. E., Dai, A., van der Schrier, G., Jones, P. D., Barichivich, J., Briffa, K. R. and Sheffield, J. (2014) 'Global warming and changes in drought', Nature Climate Change, 4(1), pp. 17–22. doi: 10.1038/NCLIMATE2067.

Vadez, V., Kholova, J., Medina, S., Kakkera, A. and Anderberg, H. (2014) 'Transpiration efficiency: New insights into an old story', Journal of Experimental Botany, 65(21), pp. 6141–6153. doi: 10.1093/jxb/eru040.

Verslues, P. E., Agarwal, M., Katiyar-Agarwal, S., Zhu, J. and Zhu, J. K. (2006) 'Methods and concepts in quantifying resistance to drought, salt and freezing, abiotic stresses that affect plant water status', The Plant Journal, 45(4), pp. 523–539. doi: 10.1111/j.1365-313X.2005.02593.x.

Whelan, T. and Sackett, W. M. (1973) 'Enzymatic fractionation of carbon isotopes by phosphoenolpyruvate carboxylase from c(4) plants.', Plant physiology, 51(6), pp. 1051–4. doi: 10.1104/pp.51.6.1051.

Wong, W. W., Benedict, C. R. and Kohel, R. J. (1979) 'Enzymic fractionation of the stable carbon isotopes of carbon-dioxide by Ribulose-1,5-Bisphosphate Carboxylase', Plant Physiology, 63, pp. 852–856.

World Economic Forum (2015) The Global Information Technology Report 2015 ICTs for Inclusive Growth.

Wright, S., Bi, I. V., Schroeder, S. G., Yamasaki, M., Doebley, J. F., McMullen, M. D. and Gaut, B. S. (2005) 'The effects of artificial selection on the maize genome', Science, 308(5726), pp. 1310–1314. doi: 10.1126/science.1107891.

WWAP (United Nations World Water Assessment Programme) (2015) The United Nations World Water Development Report 2015: Water for a Sustainable World, The UN World Water Development Report 2015. Paris: UNESCO. doi: 10.1016/S1366-7017(02)00004-1.

Zhu, M., Monroe, J. G., Suhail, Y., Villiers, F., Mullen, J., Pater, D., Hauser, F., Jeon, B. W., Bader, J. S., Kwak, J. M., Schroeder, J. I., McKay, J. K. and Assmann, S. M. (2016) 'Molecular and systems approaches towards drought-tolerant canola crops', New Phytologist, 210(4), pp. 1169–1189. doi: 10.1111/nph.13866.

Chapter 1:

Screening for Natural Variation in Water Use Efficiency Traits in a Diversity Set of *Brassica napus* L. Identifies Candidate Variants in Photosynthetic Assimilation

Abstract

Seed yield and quality of crop species are significantly reduced by water deficit. Stable isotope screening ($\delta^{13}C$) of a diversity set of 147 accessions of *Brassica napus* grown in the field identified several accessions with extremes in water use efficiency (WUE). We next conducted an investigation of the physiological characteristics of selected natural variants with high and low WUE to understand how these characteristics translate to differences in WUE. We identified an interesting Spring accession, G302 (Mozart), which exhibited the highest WUE in the field and high CO_2 assimilation rates coupled with an increased electron transport capacity (J_{max}) under the imposed conditions. Differences in stomatal density and stomatal index did not translate to differences in stomatal conductance in the investigated accessions. Stomatal conductance response to exogenous abscisic acid (ABA) was analyzed in selected high and low WUE accessions. Spring lines showed little variation in response to exogenous ABA, while one Semi-Winter line (SW047) showed a significantly more rapid response to exogenous ABA, which corresponded with the high WUE indicated by $\delta^{13}C$ measurements. This research illustrates the importance of examining natural variation at a physiological level for investigation of the underlying mechanisms of drought acclimation and identifies natural variants in *Brassica napus* with improved water use efficiency and potential relevant traits.

Introduction

Plants are exposed to a variety of environmental stresses, including drought, temperature, and salinity. The ability to cope with these stresses has a profound impact on crop productivity, with grain and seed crops losing more than half of their theoretical yield when exposed to an unfavorable environment (Boyer, 1982). The resulting decrease in crop yields translates to an economic loss and also contributes to global food insecurity. Unfortunately, both the severity and frequency of drought episodes are likely to increase as global climate change affects average global temperature and fresh water reserves are depleted (Trenberth *et al.*, 2014).

The shortage of fresh water is exacerbated both by the increasing world population and global climate change. As agricultural activities account for nearly 70% of the world's fresh water consumption, global water deficits threaten crop productivity (WWAP - United Nations World Water Assessment Programme, 2015). There is a clear need to better understand the mechanisms that underlie natural variation in plant physiology and drought tolerance. Plants have developed a variety of coping strategies to acclimate and adapt to drought stress: drought escape, dehydration avoidance and dehydration tolerance (Ludlow and Muchow, 1990). Plants can avoid dehydration by maintaining their internal water status during periods of drought by modulating water uptake through the roots (Passioura, 1983; Pinheiro *et al.*, 2005) and/or minimizing water loss through evapotranspiration by controlling stomatal conductance (Davies, Wilkinson and Loveys, 2002). There has been a wealth of detailed research on stomatal development and regulation in the model species *Arabidopsis thaliana*. Identification of

core components of the ABA signal transduction pathway in *Arabidopsis* has greatly increased our understanding of plant responses to environmental stress (Cutler *et al.*, 2010; Hauser, Waadt and Schroeder, 2011). Here we investigate the physiological characteristics that contribute to natural variation in water use efficiency in *Brassica napus*, a close relative of *Arabidopsis* (Noh and Amasino, 1999; Parkin *et al.*, 2005).

In terms of global production, *B. napus* is one of the most economically important oilseed crops for both feed stocks and fuel (FAO, 2015). Recent investigations into the evolution of *B. napus* have shown multiple allotetraploid origins of *B. napus* from hybridization of the diploid progenitors *Brassica rapa* and *Brassica oleracea*, resulting in genetic and phenotypic diversity (Allender and King, 2010). When exposed to water stress during flowering and seed setting, there is a reduction in seed yield and quality (Bouchereau *et al.*, 1996; Champolivier and Merrien, 1996; Jensen, Mogensen, Mortensen, Fieldsend, *et al.*, 1996). Crucial mechanisms and genetic loci involved in phenotypic differences may be identified by exploring natural variation between accessions of a species (Donovan and Ehleringer, 1994; Barbour *et al.*, 2010). Examining the differences in water use efficiency between diverse accessions of *B. napus* is needed to develop improved understanding of the physiological basis of variations between accessions (Zhu *et al.*, 2016).

Water use efficiency (WUE) can be defined at different scales, with integrative whole plant WUE defined as the ratio of total biomass to evapotranspiration. Intrinsic water use efficiency can be measured at the leaf level as the ratio of photosynthetic CO_2 assimilation to transpiration. Carbon isotope discrimination ($\delta^{13}C$) is used as a surrogate

for direct measurement of WUE, as discrimination against ^{13}C during photosynthesis decreases with increased water stress (Farquhar and Richards, 1984).

Here we utilized stable carbon isotope screening ($\delta^{13}C$) on a subset of 147 lines from a diversity set of 500+ accessions of field-grown *B. napus* to identify natural variation in WUE. Accessions showing predicted extremes in WUE, based on $\delta^{13}C$ data, were chosen for a detailed study of gas exchange. Using infrared gas analyzer measurements on greenhouse grown plants, we measured photosynthetic CO_2 assimilation, stomatal conductance, and transpiration efficiency of selected accessions. We also examined differences in stomatal index and density between accessions, and the responsiveness of stomatal closure to abscisic acid exposure. We determined the correlation of transpiration efficiency to the WUE determined by $\delta^{13}C$ data varied by accession, with the spring accession G302 showing an enhanced electron transport capacity and enhanced WUE based on δ13C analysis of field-grown plants, indicating that photosynthetic CO_2 assimilation rate could be a mechanism contributing to WUE in these *B. napus* accessions. We also determined differences in physiological responses between Spring-type and Semi-Winter-type accessions.

Results

Leaf carbon isotope discrimination varies in field-grown plants

We grew 147 accessions of *B. napus*, including both Spring-type (G) and Semi-Winter-type (SW) lines and both oilseed and fodder types to screen for natural variation in WUE. Plants were grown in February 2013 in the field in Maricopa, Arizona, under

irrigation. Leaf tissue was collected in April 2013, prior to flowering, to measure the carbon isotope ratio ($\delta^{13}C$), which is used as a time-integrated measure of WUE (Farquhar and Richards, 1984; Seibt *et al.*, 2008; Easlon *et al.*, 2014). Substantial variation was found in $\delta^{13}C$ between the 147 investigated accessions with a range between the extreme accessions of -30.5 ‰ (G284 Tribute) and -26.5‰ (G302 Mozart) (Figure 1A; supplemental table 1). From these field data, eight extreme accessions were chosen (4 each from Spring-type and Semi-Winter-type) representing the range of $\delta^{13}C$ values, for further physiological characterizations (Figure 1B, C). In the Spring-type accessions, G284 showed the lowest projected WUE with a $\delta^{13}C$ value of -30.5 ‰ and G302 (Mozart) had the highest projected WUE with a $\delta^{13}C$ value of -26.5 ‰ (Figure 1B). The SW accessions had a smaller range of variation than the Spring-type (G), with SW111 having the lowest WUE with an average $\delta^{13}C$ value of -29.7 ‰ and SW047 having the highest WUE with a $\delta^{13}C$ value of -26.7 ‰ (Figure 1C).

Gas exchange analyses

Instantaneous transpiration efficiency describes the ratio of the photosynthetic CO_2 assimilation rate to transpiration. Physiological characterizations of the accessions were performed to investigate whether differences in gas exchange regulation among the various accessions contributes to differences in $\delta^{13}C$ values. Steady-state gas exchange measurements were recorded under ambient CO_2 (400 ppm) and light (500 µmol photons m−2 s −1) conditions to compare variation in CO_2 assimilation rates (A) and transpiration efficiency (TE). The average photosynthetic CO_2 assimilation rates

showed little variation between lines, with the exception of line G302, which showed a slightly higher assimilation on average. The Semi-Winter line SW047 had a lower average CO_2 assimilation rate (Figure 2B) as compared to other Semi-Winter lines, however this difference was not significant. These assimilation rates translated into similar transpiration efficiency comparisons (Figure 2C, D), with G302 having a high TE which corresponds to the high photosynthetic CO_2 assimilation rate (Figure 2A) and may contribute to the high water use efficiency (Figure 1). Intrinsic water use efficiency was calculated as the relationship between photosynthetic CO_2 assimilation rates (A) and stomatal conductance (gs) (Figure 2E, F). A/g_s values in Spring lines showed a similar trend to TE, with line G302 having a higher A/g_s value as compared to other Spring lines (Figure 2E). Semi-Winter lines did not demonstrate significant differences in A/gs values (Figure 2F).

To examine whether the accessions indicate differences in biochemical limitations to photosynthesis, we examined the relationship between photosynthetic CO_2 assimilation rate and calculated internal partial pressure of CO_2 in the substomatal cavity (C_i) under saturating light. This relationship is described by a biochemical model (Farquhar, von Caemmerer and Berry, 1980) wherein CO_2 assimilation is limited by the ribulose-1,5-bisphosphate (RuBP)-saturated rate of Rubisco carboxylation under low CO_2 concentrations and by the rate of RuBP regeneration under high CO_2 concentrations (Figure 3A, B). Using this model, we calculated estimates of the maximum Rubisco carboxylation rates (V_{cmax}) and electron transport rates (J_{max}) which are related to the initial slope and plateau, respectively, of the curves in Fig. 3A, B (Table 1). In the Spring

accessions (Figure 3A), the high photosynthetic assimilation rate (*A*) of accession G302 correlated with a higher J_{max} than other Spring accessions (Table 1). Differences in the SW accessions showed SW070 had lower CO_2 assimilation rates (Figure 3B), as well as lower V_{cmax} and J_{max} values than other SW accessions (Table 1).

Effect of stomatal characteristics on transpiration efficiency

To determine if any differences in stomatal characteristics affected TE, we investigated stomatal conductance (g_s) between accessions. A similar average range of g_s was found in all lines (Figure 4A). Stomatal density (number of stomata per mm^2) and stomatal index (number of stomata/total epidermal cells) were measured for the abaxial epidermis of each line (Figure 4B, C). Lines G278 and SW070 both had significantly greater average stomatal densities and stomatal indices than other accessions (Figure 4B, C). Neither high stomatal density, as found in G278 (Figure 4D) nor lower stomatal density as found in SW111 (Figure 4E) translated to a difference in g_s (Figure 4A).

Relationship between $\delta^{13}C$ and transpiration efficiency

The instantaneous TE calculated from the ratio of photosynthetic CO_2 assimilation rates to transpiration rates was compared to the $\delta^{13}C$ of field-grown plants. In the Spring accessions the higher $\delta^{13}C$ value (high WUE) of the G302 (Mozart) line (Figure 1) was in line with an increased TE (Figure 5A). The SW lines showed a possible negative trend between $\delta^{13}C$ and TE. As TE is directly related to the CO_2 assimilation rate, this suggests the $\delta^{13}C$ value in the SW lines is related to traits other than CO_2

assimilation and transpiration rates. Experiments with these lines, where plants were grown in the field under well-watered or non-irrigated conditions, suggests field-derived $\delta^{13}C$ values may also be translated into crop performance under limited irrigation conditions for the G302 accession (Supplemental Figure 1). As the plants were grown in much different conditions in the growth room as compared to the field, we measured $\delta^{13}C$ values of growth-room plants used in the physiological assays (Figure 6 A, B). In these experiments the intermediate WUE Spring line G307, showed average $\delta^{13}C$ values that are lower than the high WUE lines G278 and G302 (Figure 6A, $P < 0.05$). Line SW070 showed average $\delta^{13}C$ values lower than the high WUE lines SW050 and SW047 (Figure 6B, $P < 0.05$). Notably, the difference in $\delta^{13}C$ values were not as pronounced in the chamber-grown plants compared to field-grown plants.

Stomatal conductance response to exogenous ABA

To investigate the effect of ABA on stomatal closure, we developed a procedure which resolves the kinetics of stomatal ABA responses in intact *B. napus* leaves, wherein we performed gas exchange analyses with ABA added to the transpiration stream. Individual leaves were excised and the petiole submerged in water. Gas exchange parameters were controlled at ambient conditions (CO_2 400 ppm, PAR 500 µmol photons m^{-2} s^{-1}) using a Li-Cor-6400 gas exchange analyzer. ABA was added to the water feeding the petioles to a final concentration of 10 µM. The stomatal conductance curves were analyzed using a standard one-phase decay equation (see Methods) to determine rate constants of stomatal closure. The difference between steady-state stomatal

conductance and final conductance ("span") was also calculated. The Spring accessions (Figure 7A-D) did not show significant differences in their rate of g_s change (Figure 7E) or span (Figure 7F). In the Semi-Winter lines (Figure 8A-D), a significant difference was observed between the rate of change of line SW047 and the other SW lines (Figure 8E). This rapid stomatal closure rate may be a reason for the $\delta^{13}C$ value recorded for accession SW047 (Figure 1), which indicated an increased WUE. Line SW070 had a significantly larger span between open and closed stomata (Figure 8F).

Discussion

Characterizing and understanding the natural variation within a species is a powerful tool to identify mechanisms and genetic loci associated with phenotypes. The work presented here demonstrates the differences in traits associated with WUE in natural variants of the crop species *B. napus* that showed extremes in WUE based on δ13C measurements.

We investigated the gas-exchange physiology of field-grown Spring and Semi-Winter *B. napus* accessions which had a range of $\delta^{13}C$ values. There is a correlation between $\delta^{13}C$ values of plant material and WUE (Farquhar and Richards, 1984). Gas exchange parameters of photosynthetic carbon assimilation (A) and stomatal conductance (g_s) were measured in selected accessions, with no significant difference found in most accessions. Interestingly, the Spring line G302 (Mozart), which had the highest WUE in the field, had a high rate of CO_2 assimilation (Figure 3A) which translated to a high TE. Analysis of additional accessions would be needed to infer a correlation

between $\delta^{13}C$ values and instantaneous TE. Differences in stomatal density and stomatal index did not translate to altered stomatal conductance or assimilation in the investigated extreme WUE accessions, which differs from results in previous transgenic studies in *Arabidopsis* (Doheny-Adams *et al.*, 2012). The difference in $\delta^{13}C$ values observed in field-grown versus growth room plants highlights the growth condition dependence of $\delta^{13}C$ values.

Our study also examined the stomatal conductance response of these accessions to exogenous ABA. Examining the kinetics of plant responses to ABA in intact leaves allowed us to investigate the relationship of WUE to the rate of stomatal response to ABA. We were able to elicit stomatal closure upon addition of ABA to the transpiration stream in all accessions tested. Spring lines exhibited no significant difference in their rate of stomatal closure or the span of difference in stomatal conductance before and after ABA treatment. The Semi-Winter line SW047 had a significantly faster rate of stomatal closure as compared to the other SW lines, which may correlate with the higher WUE indicated by the $\delta^{13}C$ value. Line SW070 had a significantly larger span of g_s before and after ABA treatment compared to other SW lines.

Recent studies have demonstrated cuticle permeability to both water vapor and CO_2 as having a contribution to water loss from plants (Boyer, 2015a, 2015b), particularly in leaves with closed stomata (Tominaga and Kawamitsu, 2015). As the cuticle allows water vapor to exit the leaf at a higher rate than CO_2 can enter, this can impact the difference between calculated CO_2 flux and actual CO_2 entering the leaf (Hanson, Stutz and Boyer, 2016). Analysis of the cuticle composition of the *B. napus*

diversity set, from which the accessions used in this study, revealed heritable variation in cuticular wax composition and amount (Tassone *et al.*, 2016). The Tassone et al. study identified relatively high amounts of *n*-alkanes, which have been linked to the inhibition of leaf water loss in previous studies (Leide *et al.*, 2007; Kosma *et al.*, 2009). Measurements of the cuticle composition and cuticular wax amount in the studied accessions under well-watered and water-stressed conditions may indicate whether these traits contribute to the long term water use efficiency observed in these accessions.

Conclusions

This study shows how characterization of natural variation in $\delta^{13}C$ derived WUE within a species provides an approach for understanding the many traits involved in WUE phenotypes. The present study indicates the Semi-Winter line SW047 shows a more rapid ABA response which may be linked to WUE and the spring line G302 had an increased electron transport capacity (J_{max}), which may also be linked to the higher WUE. These results could be used to further examine the mechanisms and genetic differences between these accessions and shows the potential of using this diversity set to characterize mechanisms that affect WUE.

Materials and methods

Plant material and growth conditions

Growth room experiments. *B. napus* seeds were sown in 3-inch pots containing a mixture of potting soil, perlite and vermiculite (6:1:1) and placed in a walk-in growth room at a controlled temperature (22°C) and humidity (60 ± 2% RH) with a 12-h light:12-h dark regime at 150 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD). Seedlings were watered every other day to soil capacity. Three weeks after germination, plants were transferred to 5-inch pots containing the same soil mixture. Plants were grown at controlled temperature (22°C) and humidity (60 ± 2% RH) with a 12-h light:12-h dark regime at 250 µmol m^{-2} s^{-1} PPFD at canopy level. Plants were watered to soil capacity every other day. Experiments were performed between January 2014 and June 2015, with seeds sown of each line every 4-6 weeks, for continuous availability of plants for measurement. All plants were 6-8 weeks old at time of measurements.

Drought tolerance experiments. The diversity set lines at the Colorado State University Agricultural Research, Development and Education Center were grown near Fort Collins, Colorado (40.65°N, 105.00°W). The soil type was Nunn clay loam, and average annual precipitation was 356 mm. Seeds were planted in a split-plot design with a well-irrigated and a drought treatment and three replicates in May 2015. Plots were 1.5 m by two rows, with 0.3-m row spacing. Irrigation was applied using a linear-move system at approximately 2.5 cm per week for the first 7 weeks of development at which point it was discontinued in the drought treatment. Irrigation was maintained at the rate of 2.5 cm per week for the duration of the experiment in the irrigated treatment. At seed maturity, all plants were cut at soil level and plot-level aerial fresh biomass was measured.

Carbon isotope discrimination

The diversity set of *B. napus* (Supplemental Table 1) was grown in 3-m, one-row plots with three replicates in an α-lattice design at the Maricopa Agricultural Center of the University of Arizona in Maricopa, Arizona, as described by (Tassone *et al.*, 2016). Soil is a Casa Grande sandy loam and plants were flood irrigated. Seeds were sown in early February 2013. At eight weeks from planting, we sampled 147 accessions, taking two non-shaded leaves from a random plant collected from each plot. Leaf tissue was dried at 65 °C for 48 h and then crushed. Aliquots containing 2-mg of leaf tissue were used to quantify carbon isotope ratio ($\delta^{13}C$, expressed relative to the Vienna PeeDee Belemnite standard) using a dual-inlet mass spectrometer (PDZ Europa 20-20 isotope ratio mass spectrometer, PDZ Europa ANCA-GSL elemental analyzer, Sercon Ltd., Cheshire, UK) at the Stable Isotope facility at University of California, Davis. Samples for growth room plants were collected similarly to field-grown plants, collecting three cauline leaves from each plant after bolting and prior to seed setting. Aliquots containing 2-mg of leaf tissue were used to quantify carbon isotope ratio ($\delta^{13}C$, expressed relative to the Vienna PeeDee Belemnite standard) using a dual-inlet mass spectrometer (Delta V mass spectrometer, Conflo IV interface, Thermo Scientific, Waltham MA, USA; ECS 4010 CHNSO Analyzer, Costech Analytical Technologies, Inc., Valencia CA, USA) at the Center for Stable Isotopes at University of New Mexico.

Physiological analyses

Gas exchange measurements from intact, mature leaves of 6-8 week old plants were conducted using a LI-6400 infrared gas exchange analyzer (LI-6400XT, Li-Cor, Inc., Lincoln NE, USA) with the standard 6 cm^2 leaf cuvette fitted with an LED light source (LI-6400-02B; Li-Cor Inc.). Leaf temperature and vapor pressure deficit at the leaf level (VpdL) were held at 20°C and ~0.75 kPa (±0.05 kPa), respectively (Supplemental Figure 2). All measurements were taken at 500 µmol m^{-2} s^{-1} PPFD (intensity determined to be at light saturation for all accessions using standard light response curve at 400 ppm CO_2). Steady-state gas exchange measurements (*A*, *gs*, *E*) were taken at 400 ppm CO_2. Photosynthetic parameters (J_{max}, Vc_{max}) were estimated from A/Ci curves according to the method of Sharkey et al (2007). Values normalized to leaf temperature 25°C.

Stomatal ABA response analysis

Intact, mature leaves of 6-8 week old plants were removed and the petiole cut under water 2 cm from the base of the leaf. The cut end was submerged in deionized H_2O in a 15-mL Falcon tube. Gas exchange measurements were conducted as above with the LI-6400XT gas exchange analyzer. Leaf temperature and relative humidity were held at 20°C and ~75% (±5%), respectively. Light intensity for measurements was 500 µmol m^{-2} s^{-1} PPFD, and reference [CO_2] set at 400 ppm. After ten minutes of steady state or more stable CO_2 assimilation rates (*A*) and stomatal conductance (g_s), ABA was added to the Falcon tube to a final concentration of 10 µM. Gas exchange data were collected for 30 minutes after the addition of ABA. In control experiments 15µL ethanol was added in place of ABA (data not shown). Curves were analyzed using GraphPad Prism.

Rate of change (K) and span were determined by fitting a plateau followed by one-phase decay algorithm (Model: Y= IF(X<X0, Y0, Plateau+(Y0-Plateau)*exp(-K*(X-X0))) where X0 is the time at which the decay begins and Y0 is the average Y value prior to time X0). Differences in K and span within each group (SW or G) were analyzed with one-way ANOVA followed by Tukey's multiple comparisons test.

Stomatal density/index analyses

Following gas exchange measurements, three 1 cm diameter punches were taken from the area of leaf that was used for gas exchange. The punches were stained with propidium iodide (100 µg/mL) for one hour, then rinsed with distilled H_2O and transferred to slides. Confocal microscopy was performed using a custom spinning disk confocal microscope system described previously (Walker et al., 2007). Laser excitation was 568 nm for propidium iodide. Images were acquired and Z-stack projections assembled using MetaMorph software (Universal Imaging). Image processing was performed using NIH ImageJ. Data within each group (SW or G) were analyzed with one-way ANOVA followed by Tukey's multiple comparisons test.

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REFERENCES

Allender, C. J. and King, G. J. (2010) 'Origins of the amphiploid species Brassica napus L. investigated by chloroplast and nuclear molecular markers', BMC Plant Biology, 10, p. 54. doi: 10.1186/1471-2229-10-54.

Barbour, M. M., Warren, C. R., Farquhar, G. D., Forrester, G. and Brown, H. (2010) 'Variability in mesophyll conductance between barley genotypes, and effects on transpiration efficiency and carbon isotope discrimination.', Plant, Cell & Environment, 33(7), pp. 1176–85. doi: 10.1111/j.1365-3040.2010.02138.x.

Bouchereau, A., Clossais-Besnard, N., Bensaoud, A., Leport, L. and Renard, M. (1996) 'Water stress effects on rapeseed quality', European Journal of Agronomy, 5, pp. 19–30.

Boyer, J. S. (1982) 'Plant productivity and environment.', Science, 218(4571), pp. 443–448. doi: 10.1126/science.218.4571.443.

Boyer, J. S. (2015a) 'Impact of cuticle on calculations of the CO2 concentration inside leaves', Planta. Springer Berlin Heidelberg, 242(6), pp. 1405–1412. doi: 10.1007/s00425-015-2378-1.

Boyer, J. S. (2015b) 'Turgor and the transport of CO2 and water across the cuticle (epidermis) of leaves', Journal of Experimental Botany, 66(9), pp. 2625–2633. doi: 10.1093/jxb/erv065.

Champolivier, L. and Merrien, A. (1996) 'Effects of water stress applied at different growth stages to Brassica napus L. var. oleifera on yield, yield components and seed quality', 5, pp. 153–160.

Cutler, S. R., Rodriguez, P. L., Finkelstein, R. R. and Abrams, S. R. (2010) 'Abscisic acid: emergence of a core signaling network', Annual Reviews of Plant Biology, 61, pp. 651–679.

Davies, W. J., Wilkinson, S. and Loveys, B. (2002) 'Stomatal control by chemical signalling and the exploitation of this mechanism to increase water use efficiency in agriculture', New Phytologist, 153(3), pp. 449–460. doi: 10.1046/j.0028-646X.2001.00345.x.

Doheny-Adams, T., Hunt, L., Franks, P. J., Beerling, D. J. and Gray, J. E. (2012) 'Genetic manipulation of stomatal density influences stomatal size, plant growth and tolerance to restricted water supply across a growth carbon dioxide gradient', Philosophical Transactions of The Royal Society, 367, pp. 547–555. doi: 10.1098/rstb.2011.0272.

Donovan, L. A. and Ehleringer, J. R. (1994) 'Carbon isotope discrimination, water-use efficiency, growth, and mortality in a natural shrub population', Oecologia, 100(3), pp. 347–354. doi: 10.1007/bf00316964.

Easlon, H. M., Nemali, K. S., Richards, J. H., Hanson, D. T., Juenger, T. E. and McKay, J. K. (2014) 'The physiological basis for genetic variation in water use efficiency and carbon isotope composition in Arabidopsis thaliana.', Photosynthesis research, 119(1), pp. 119–129. doi: 10.1007/s11120-013-9891-5.

FAO (2015) Food Outlook, Biannual report on global food markets. Rome.

Farquhar, G. D., von Caemmerer, S. and Berry, J. A. (1980) 'A biochemical model of photosynthetic CO2 assimilation in leaves of C3 species', Planta, 149(1), pp. 78–90. doi: 10.1007/BF00386231.

Farquhar, G. D. and Richards, R. A. (1984) 'Isotopic composition of plant carbon correlates with water-use efficiency of wheat genotypes', Australian Journal of Plant Physiology, 11(1), pp. 539–552.

Hanson, D. T., Stutz, S. S. and Boyer, J. S. (2016) 'Why small fluxes matter: The case and approaches for improving measurements of photosynthesis and (photo)respiration', Journal of Experimental Botany, 67(10), pp. 3027–3039. doi: 10.1093/jxb/erw139.

Hauser, F., Waadt, R. and Schroeder, J. I. (2011) 'Evolution of abscisic acid synthesis and signaling mechanisms.', Current Biology. Elsevier Ltd, 21(9), pp. R346-55. doi: 10.1016/j.cub.2011.03.015.

Jensen, C. R., Mogensen, V. O., Mortensen, G., Fieldsend, J. K., Milford, G. F. J., Andersen, M. N. and Thage, J. H. (1996) 'Seed glucosinolate, oil and protein contents of field-grown rape (Brassica napus L.) affected by soil drying and evaporative demand', Field Crops Research, 47, pp. 93–105.

Kosma, D. K., Bourdenx, B., Bernard, A., Parsons, E. P., Lü, S., Joubès, J. and Jenks, M. A. (2009) 'The impact of water deficiency on leaf cuticle lipids of Arabidopsis.', Plant Physiology, 151(4), pp. 1918–29. doi: 10.1104/pp.109.141911.

Leide, J., Hildebrandt, U., Reussing, K., Riederer, M. and Vogg, G. (2007) 'The developmental pattern of tomato fruit wax accumulation and its impact on cuticular transpiration barrier properties: effects of a deficiency in a beta-ketoacyl-coenzyme A synthase (LeCER6).', Plant Physiology, 144(3), pp. 1667–79. doi: 10.1104/pp.107.099481.

Ludlow, M. M. and Muchow, R. C. (1990) 'A critical evaluation of traits for improving crop yields in water-limited environments', Advances in Agronomy, 43(C), pp. 107–153. doi: 10.1016/S0065-2113(08)60477-0.

Noh, Y.-S. and Amasino, R. M. (1999) 'Regulation of developmental senescence is conserved between Arabidopsis and Brassica napus', Plant Molecular Biology, 41(2), pp. 195–206. doi: 10.1023/A:1006389803990.

Parkin, I. A. P., Gulden, S. M., Sharpe, A. G., Lukens, L., Trick, M., Osborn, T. C. and Lydiate, D. J. (2005) 'Segmental structure of the Brassica napus genome based on comparative analysis with Arabidopsis thaliana.', Genetics, 171(2), pp. 765–81. doi: 10.1534/genetics.105.042093.

Passioura, J. B. (1983) 'Roots and drought resistance', Agricultural Water Management, 7(1–3), pp. 265–280. doi: 10.1016/0378-3774(83)90089-6.

Pinheiro, H. A., DaMatta, F. M., Chaves, A. R. M., Loureiro, M. E. and Ducatti, C. (2005) 'Drought tolerance is associated with rooting depth and stomatal control of water use in clones of Coffea canephora', Annals of Botany, 96(1), pp. 101–108. doi: 10.1093/aob/mci154.

Seibt, U., Rajabi, A., Griffiths, H. and Berry, J. A. (2008) 'Carbon isotopes and water use efficiency: Sense and sensitivity', Oecologia, 155(3), pp. 441–454. doi: 10.1007/s00442-007-0932-7.

Tassone, E. E., Lipka, A. E., Tomasi, P., Lohrey, G. T., Qian, W., Dyer, J. M., Gore, M. A. and Jenks, M. A. (2016) 'Chemical variation for leaf cuticular waxes and their levels revealed in a diverse panel of Brassica napus L.', Industrial Crops and Products. Elsevier B.V., 79, pp. 77–83. doi: 10.1016/j.indcrop.2015.10.047.

Tominaga, J. and Kawamitsu, Y. (2015) 'Cuticle affects calculations of internal CO2 in leaves closing their stomata', Plant and Cell Physiology, 56(10), pp. 1900–1908. doi: 10.1093/pcp/pcv109.

Trenberth, K. E., Dai, A., van der Schrier, G., Jones, P. D., Barichivich, J., Briffa, K. R. and Sheffield, J. (2014) 'Global warming and changes in drought', Nature Climate Change, 4(1), pp. 17–22. doi: 10.1038/NCLIMATE2067.

WWAP (United Nations World Water Assessment Programme) (2015) The United Nations World Water Development Report 2015: Water for a Sustainable World, The UN World Water Development Report 2015. Paris: UNESCO. doi: 10.1016/S1366-7017(02)00004-1.

Zhu, M., Monroe, J. G., Suhail, Y., Villiers, F., Mullen, J., Pater, D., Hauser, F., Jeon, B. W., Bader, J. S., Kwak, J. M., Schroeder, J. I., McKay, J. K. and Assmann, S. M. (2016) 'Molecular and systems approaches towards drought-tolerant canola crops', New Phytologist, 210(4), pp. 1169–1189. doi: 10.1111/nph.13866.

TABLES

Table 1. Maximum Rubisco carboxylation rates (V_{cmax}) and Electron transport capacity (J_{max}) derived from the theoretical relationships shown in **Figure 3.** Parameters were estimated according to the method of Sharkey et al (2007) by fitting the model to measured A/C_i values. Values were normalized to 25°C leaf temperature

Genotype	**V_{cmax}**	**J_{max}**
	(µmol C m^{-2} s^{-1})	**(µmol e^- m^{-2} s^{-1})**
G278	54.48866	105.1209
G284	64.16738	95.89537
G302	86.66855	126.1483
G307	53.47971	90.8347
SW047	46.65277	91.00118
SW050	31.64616	69.4256
SW070	32.37564	49.6159
SW111	52.05389	94.35183

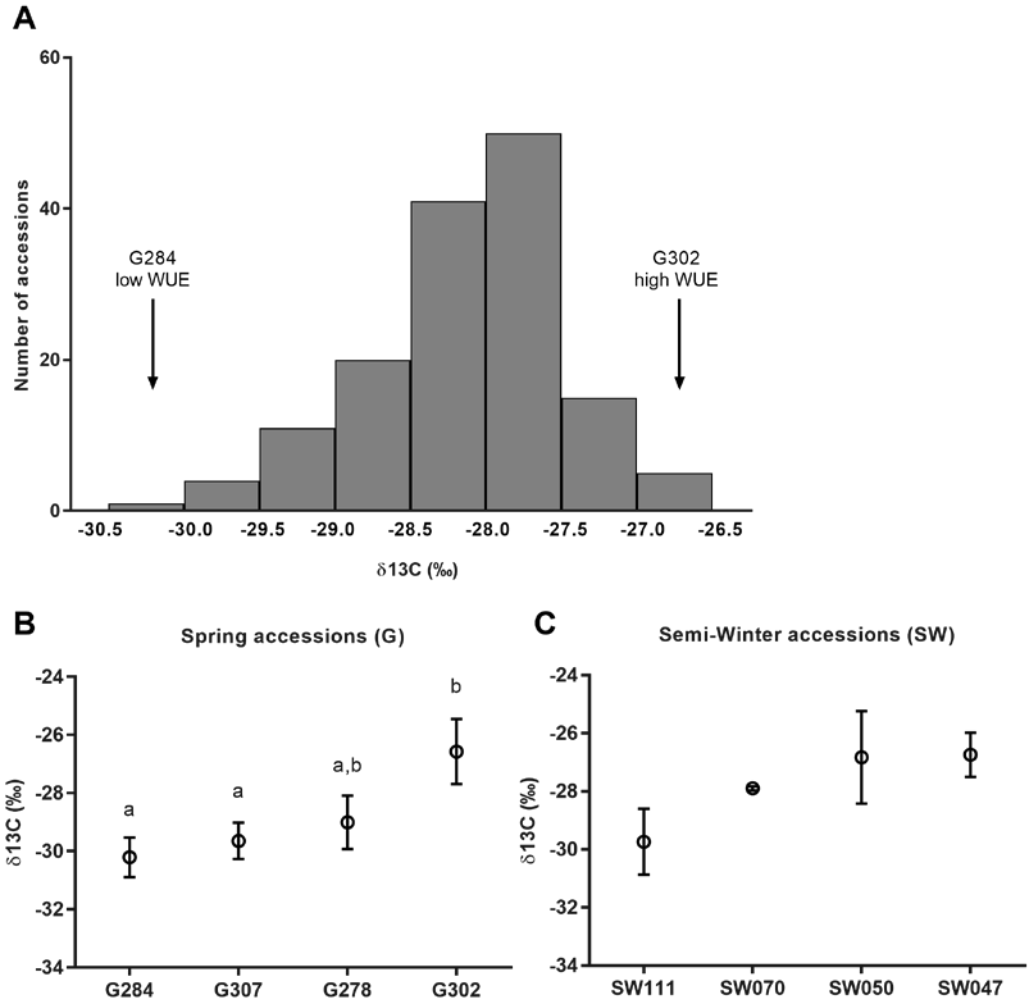


Figure 1. Leaf carbon isotope discrimination ($\delta^{13}C$) in field-grown plants. Diverse Spring (G) and Semi-Winter (SW) accessions of *Brassica napus* were grown in the field in Maricopa, Arizona, under irrigation. **A)** Leaf tissue was collected prior to flowering, and $\delta^{13}C$ was measured. A wide variation in $\delta^{13}C$ was found between accessions. **B, C)** $\delta^{13}C$ of selected accessions including accessions with higher water use efficiency (G302) and lower water use efficiency (G284). Error bars denote s.e.m. Statistical values for differences within categories were calculated using a one-way ANOVA followed by Tukey's multiple comparisons test.

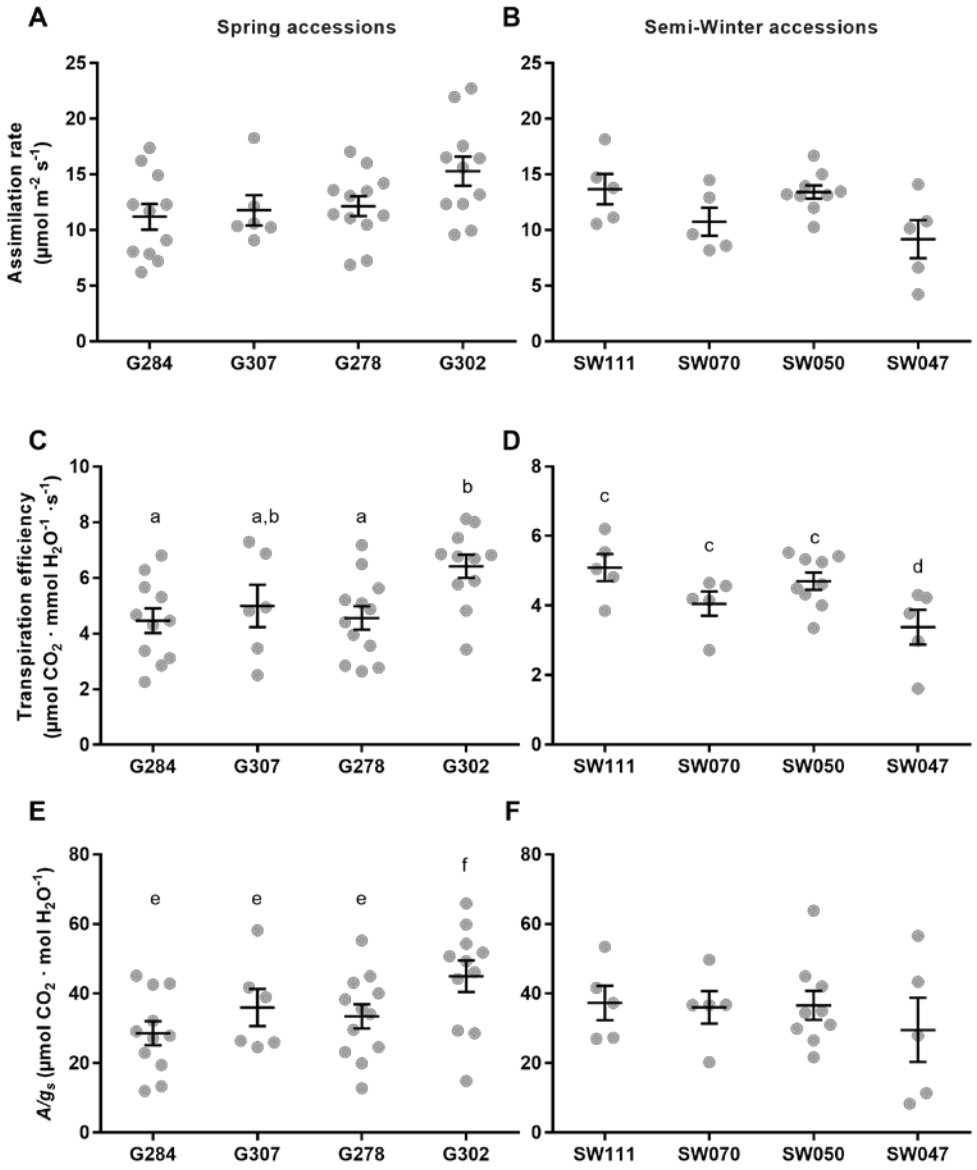


Figure 2. Physiological responses of *B. napus* under ambient conditions. Photosynthetic assimilation of Spring accessions **(A)** and Semi-Winter accessions **(B)** was recorded under ambient CO_2 (400 ppm) and light (PAR 500 µmol photons m^{-2} s^{-1}) conditions. **C, D)** Instantaneous transpiration efficiency was calculated as the ratio of photosynthetic CO_2 assimilation rate to transpiration rate. E, F) Intrinsic transpiration efficiency was calculated as the ratio of photosynthetic CO_2 assimilation rate to stomatal conductance. Error bars denote s.e.m. Statistical values for differences within categories were calculated using a one-way ANOVA followed by Tukey's multiple comparisons test.

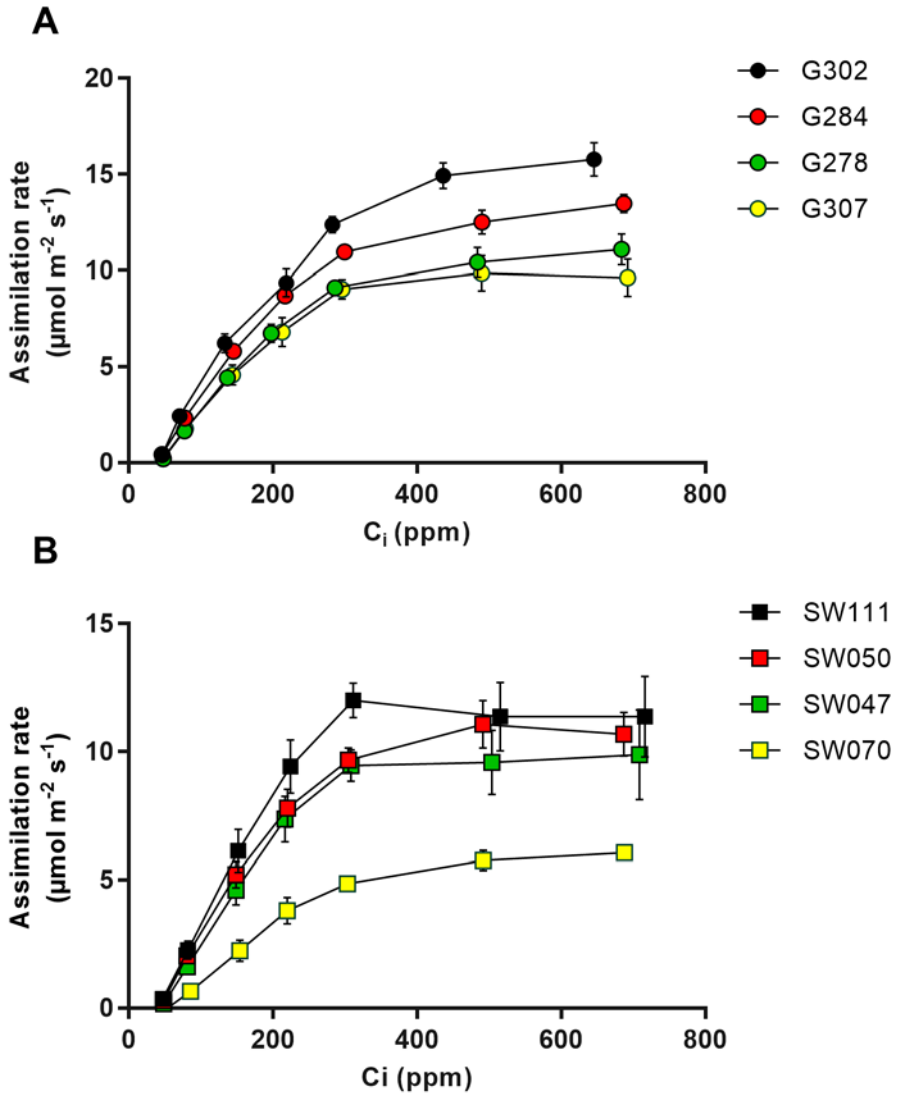


Figure 3. Analyses of CO2 assimilation rates as a function of Ci. Relationships between photosynthetic CO2 assimilation rate, measured under saturating light, and internal partial pressure of CO2 in the substomatal cavity for **A)** Spring accessions and **B)** Semi-Winter accessions. Error bars denote s.e.m., n=3.

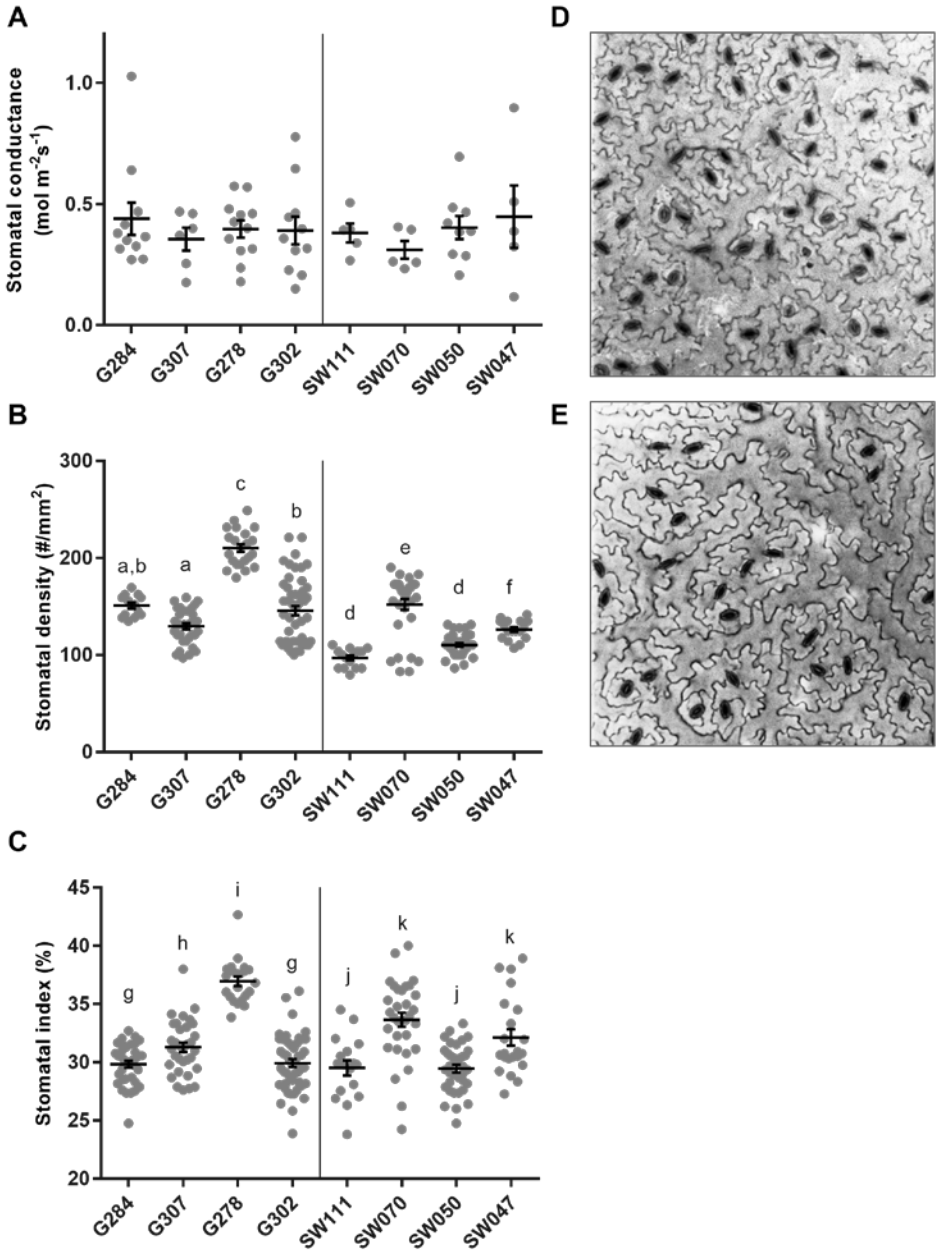


Figure 4. Effect of stomatal features on transpiration efficiency. A) Steady-state stomatal conductance calculated from gas exchange measurements on mature leaves. **B)** Stomatal densities (abaxial epidermis) **C)** Stomatal index (abaxial epidermis). Error bars denote s.e.m. Statistical values for differences within categories were calculated using a one-way ANOVA followed by Tukey's multiple comparisons test. **D-E)** Examples of stomatal densities (G278 – high stomatal density; SW111 – low stomatal density). Confocal images of abaxial epidermis stained with propidium iodide.

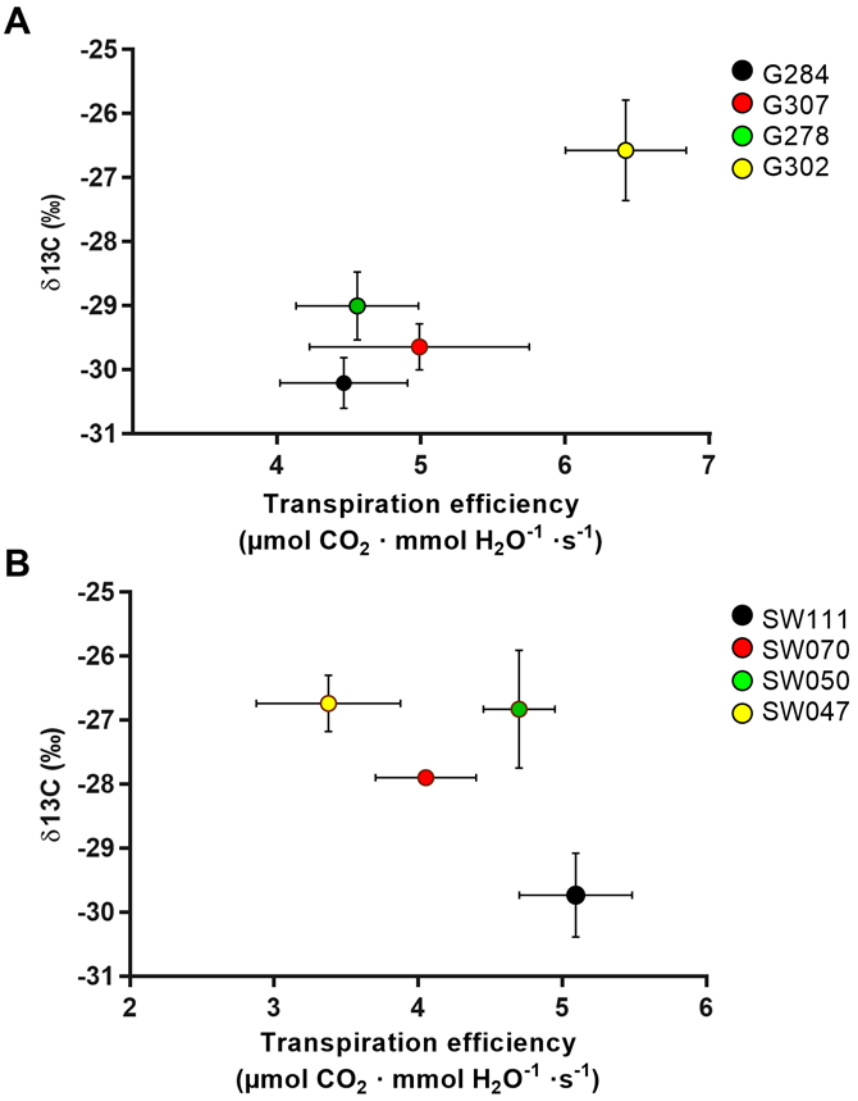


Figure 5. Relationship between δ13C and calculated transpiration efficiency. A) Spring lines show a positive trend between δ13C and transpiration efficiency determined by the ratio of photosynthetic assimilation and transpiration rate. (r2 = 0.866) B) Semi-Winter lines did not show a correlation.

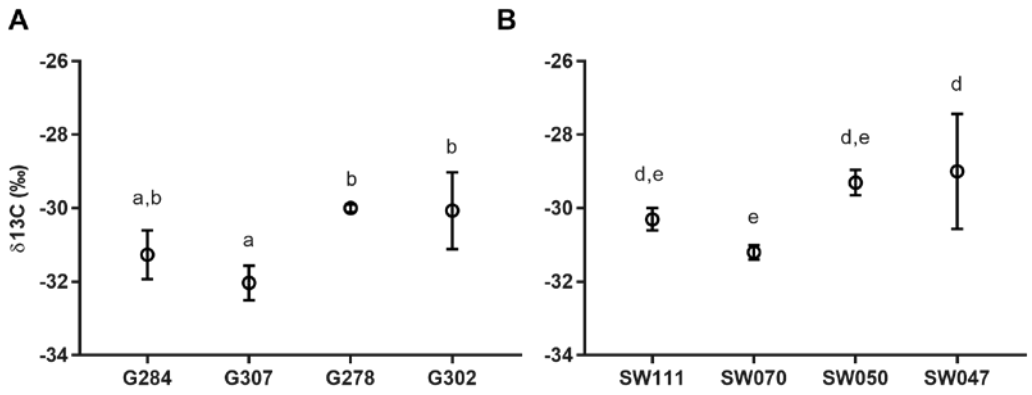


Figure 6. δ13C values of walk-in growth room plants. Carbon isotope data were collected for plants grown in the growth room. A) Spring lines. B) Semi-Winter lines (means ± s.e.m; n=3). Statistical values for differences within categories were calculated using a one-way ANOVA followed by Tukey's multiple comparisons test.

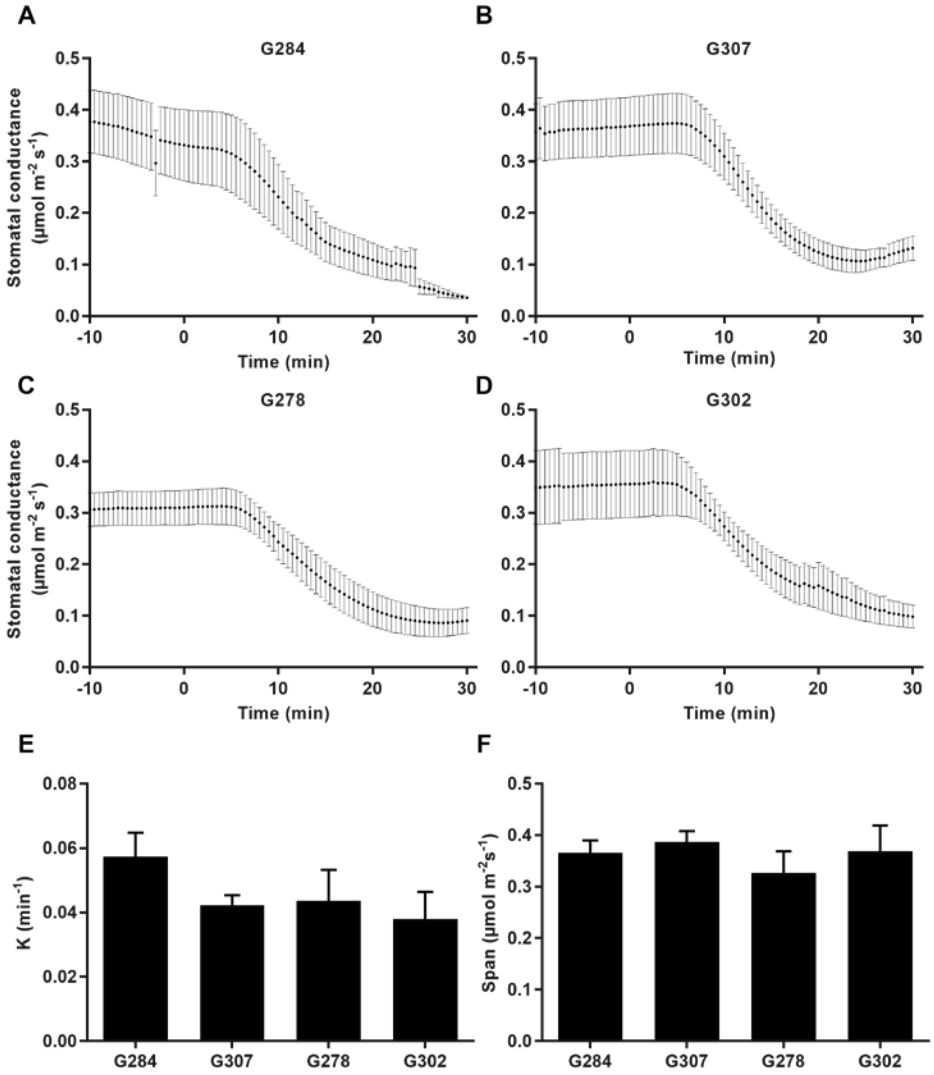


Figure 7. Stomatal conductance response to exogenous ABA in Spring accessions. Individual leaves were excised and the petiole submerged in water. Gas exchange parameters were controlled at ambient conditions (CO2 400 ppm, PAR 500 µmol photons m−2s−1) using a Li-Cor-6400 gas exchange analyzer. When steady stomatal conductance was observed, ABA was added to the transpiration stream to a final concentration of 10 µM. A-D) ABA response curves of Spring accessions to 10 µM ABA (means ± s.e.m; n=3). No significant difference was found in the E) rate constant (K) or F) difference between starting and ending stomatal conductance values (span), between Spring lines. Curves were fitted, and rate constant (K) and span determined, using a standard one-phase decay equation. Statistical values for differences within categories were calculated using a one-way ANOVA followed by Tukey's multiple comparisons test.

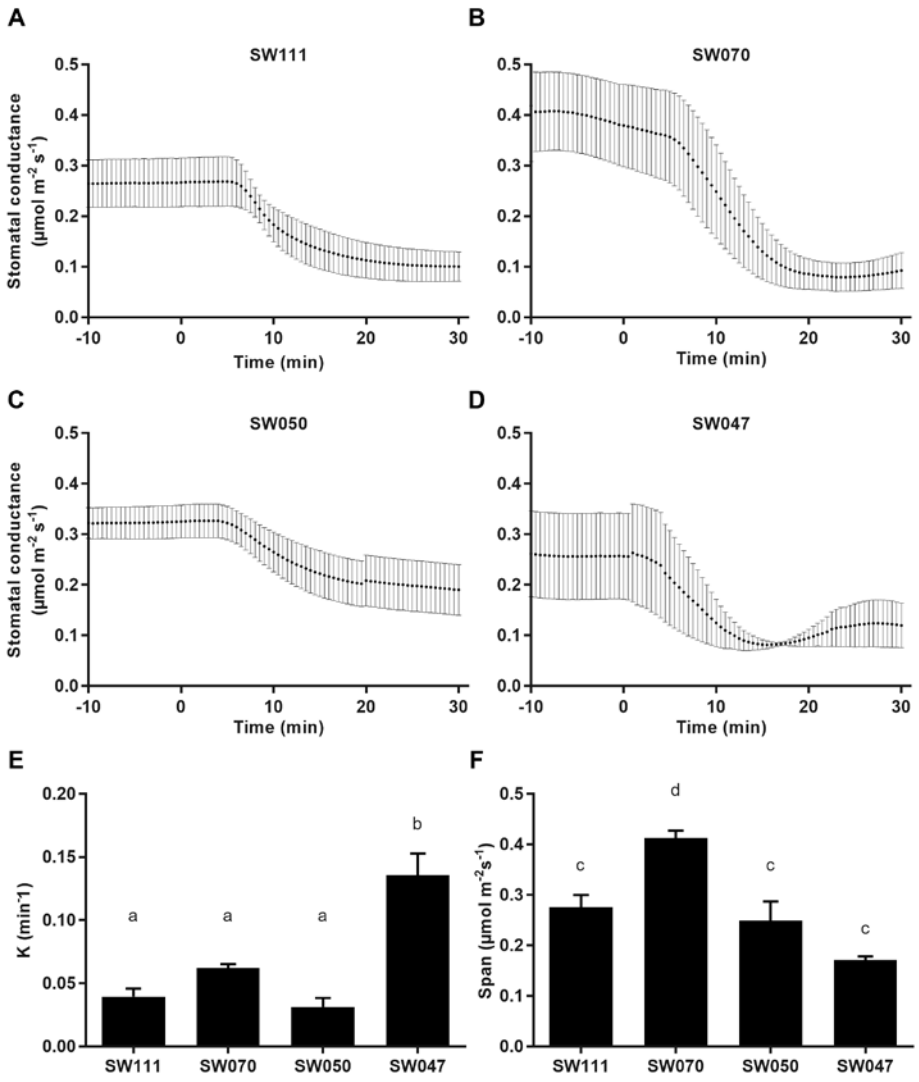
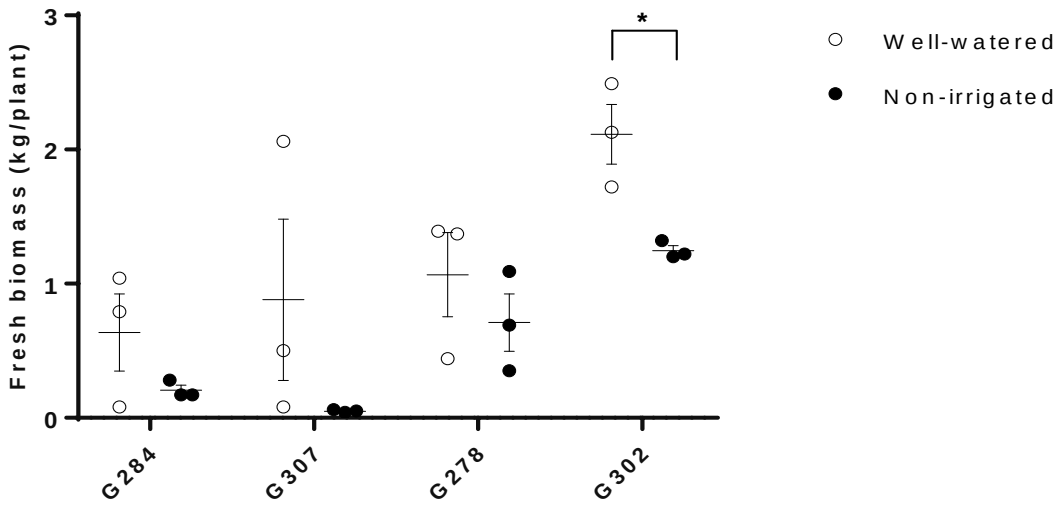


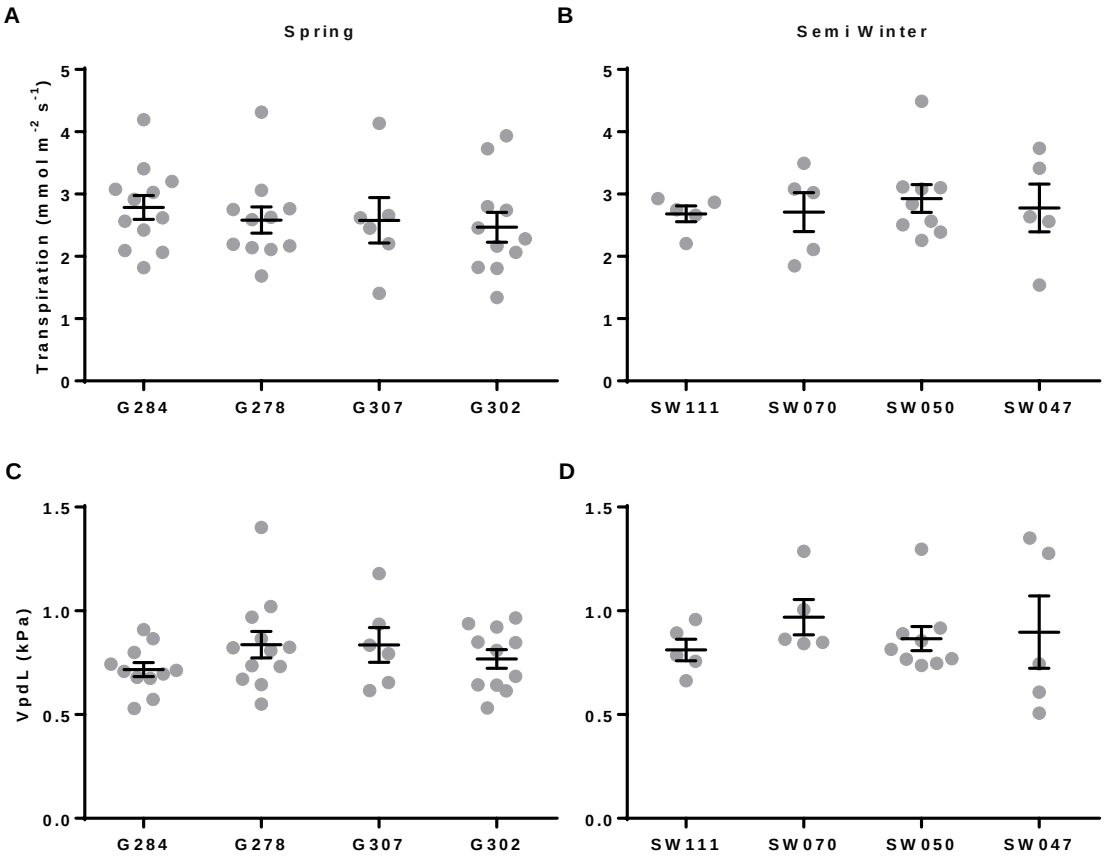
Figure 8. Stomatal conductance response to exogenous ABA. Measurements were conducted as in Figure 7. A-D) ABA response curves of Semi-Winter accessions to 10 µM ABA (means ± s.e.m; n=3). In Semi-Winter accessions, E) SW047 had a significantly higher rate constant than other accessions. F) SW070 had a significantly larger span than other Semi-Winter accessions. Curves were fit, and rate constant (K) and span (difference between starting and ending stomatal conductance values) determined, using a standard one-phase decay equation. Statistical values for differences within categories were calculated using a one-way ANOVA followed by Tukey's multiple comparisons test.

Fig S1

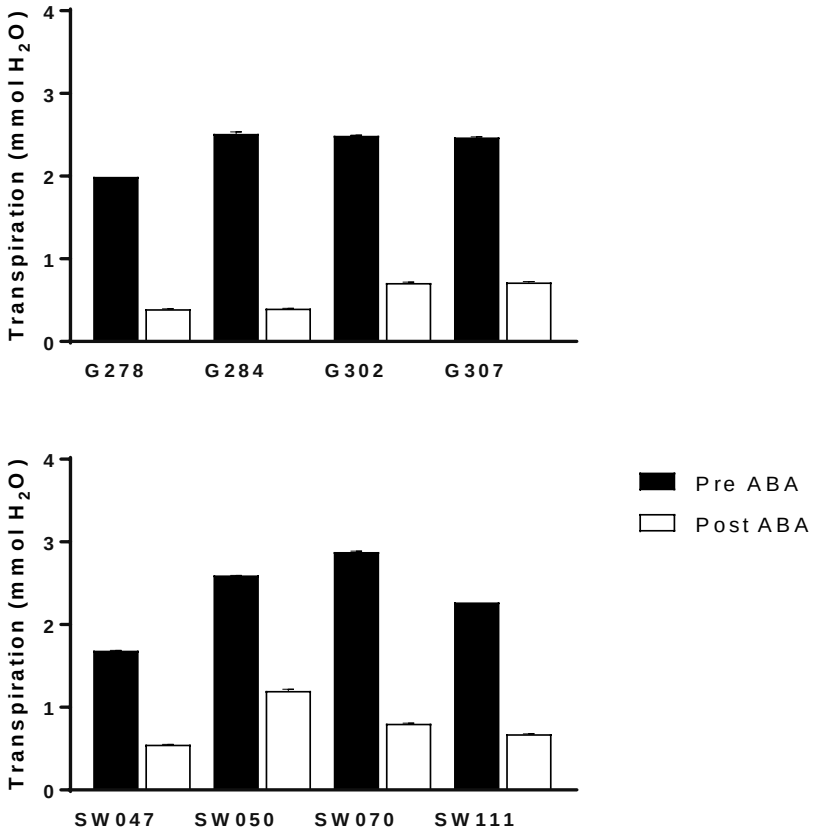


S1. Fresh biomass of field-grown Spring lines under well-watered (white symbols) and non-irrigated (black symbols) conditions. Plants were harvested after seed-set and fresh shoot mass was recorded. Statistical values for differences between treatments were calculated using a two-way ANOVA followed by Fisher's LSD test. ($P < 0.05$; n=3)

Fig S2



S2. Transpiration rates of (A) Spring and (B) Semi-Winter accessions. Error bars denote s.e.m. Statistical values for differences within categories were calculated using a one-way ANOVA followed by Tukey's multiple comparisons test and showed no significant differences between accessions.



S3. Transpiration rates in response to exogenous ABA. Bars represent average of 10 minutes of measurements prior to ABA addition (black bars) and 10 minutes of low transpiration data after ABA addition (white bars). (means ± s.e.m.; n=5). No significant differences were found between post ABA values amongst accessions. Statistical values for differences within categories were calculated using a two-way ANOVA followed by Sidak's multiple comparisons test.

Appendix A

Molecular and systems approaches towards drought-tolerant canola crops

New Phytologist

Review

Tansley review

Molecular and systems approaches towards drought-tolerant canola crops

Author for correspondence:
Sarah M. Assmann
Tel: +1 814 863 9579
Email: sma3@psu.edu

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Mengmeng Zhu[1], J. Grey Monroe[2], Yasir Suhail[3], Florent Villiers[4], Jack Mullen[2], Dianne Pater[5], Felix Hauser[5], Byeong Wook Jeon[1], Joel S. Bader[3,6], June M. Kwak[4,7], Julian I. Schroeder[5], John K. McKay[2] and Sarah M. Assmann[1]

[1]Biology Department, Pennsylvania State University, University Park, PA 16802, USA; [2]Bioagricultural Sciences and Pest Management, Colorado State University, Fort Collins, CO 80523, USA; [3]Department of Biomedical Engineering, The Johns Hopkins School of Medicine, Baltimore, MD 21205, USA; [4]Department of Cell Biology and Molecular Genetics, University of Maryland, College Park, MD 20740, USA; [5]Division of Biological Sciences, Cell and Developmental Biology Section, Food and Fuel for the 21st Century Center, University of California San Diego, La Jolla, CA 92093-016, USA; [6]School of Medicine, The Johns Hopkins University, Baltimore, MD 21205, USA; [7]Present address: Center for Plant Aging Research, Institute for Basic Science, Department of New Biology, DGIST, Daegu 42988, Korea

Contents

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Key words: abscisic acid (ABA), *Arabidopsis thaliana*, *Brassica napus*, canola, drought, oilseeds, natural variation, translational biology.

Summary

Modern agriculture is facing multiple challenges including the necessity for a substantial increase in production to meet the needs of a burgeoning human population. Water shortage is a deleterious consequence of both population growth and climate change and is one of the most severe factors limiting global crop productivity. *Brassica* species, particularly canola varieties, are cultivated worldwide for edible oil, animal feed, and biodiesel, and suffer dramatic yield loss upon drought stress. The recent release of the *Brassica napus* genome supplies essential genetic information to facilitate identification of drought-related genes and provides new information for agricultural improvement in this species. Here we summarize current knowledge regarding drought responses of canola, including physiological and -omics effects of drought. We further discuss knowledge gained through translational biology based on discoveries in the closely related reference species *Arabidopsis thaliana* and through genetic strategies such as genome-wide association studies and analysis of natural variation. Knowledge of drought tolerance/resistance responses in canola together with research outcomes arising from new technologies and methodologies will inform novel strategies for improvement of drought tolerance and yield in this and other important crop species.

I. Introduction

Fresh water scarcity is an emerging global problem, and given that the majority of fresh water extracted by humans is used for agriculture (Rosegrant *et al.*, 2009), improving crop production under limited water availability is an important challenge. Although crop production can be enhanced by water conservation through improvements in tillage and irrigation practices, modification of the genetic basis of stress tolerance in crops is an urgently needed complementary strategy for improving productivity under conditions of moisture deficit (Turner, 2001; Pennisi, 2008). It is estimated that crops attain less than half of their potential yield as a result of unfavorable environmental conditions, with water deficit being the most severe stress (Boyer, 1982; Gleick, 1998; Araus *et al.*, 2002). Given climate change scenarios, drought tolerance will be an increasingly necessary agronomic characteristic.

There are over 3000 species within the *Brassicaceae* (mustard family) and they are mainly cultivated in the northern hemisphere. The *Brassicaceae* includes many familiar vegetable crops (e.g. broccoli, cauliflower, Chinese cabbage, and various mustards). Also included in the *Brassicaceae* are the reference plant, *Arabidopsis thaliana*, and the oilseed crops, particularly *Brassica napus* (Al-Shehbaz, 1984). *Brassica* species provide *c.* 12% of the edible oil worldwide, particularly from the canola varieties (Paterson *et al.*, 2001; Hall *et al.*, 2002). Standing for Canada (Can) oil (ola), the word 'canola' refers to types of rapeseed varieties originally developed in Canada for edible oil, animal feed, and biodiesel, with low glucosinolate and erucic acid content (http://www.canolacouncil.org/). Canola quality oil is derived from three species: *B. napus*, *Brassica rapa*, and *Brassica juncea*. Among the canola species, *B. napus*, an amphidiploid species (AC genome, $n = 19$), is derived from a recent (presumably < 10 000 yr ago) hybridization of *B. rapa* (A genome, $n = 10$) and *Brassica oleracea* (C genome, $n = 9$) (Palmer *et al.*, 1983; Wan *et al.*, 2009; Schmidt & Bancroft, 2011; Wang *et al.*, 2011a).

Brassica napus possesses favorable agronomic properties; for example, cultivation under different seasons (annuals and biennials) and rotation with cereals is possible. *B. napus* produces high-quality oil (Ahmadi, 2010) and is currently the third largest source of global vegetable oil supplies, after soybean and palm (http://faostat3.fao.org). During the past decade, annual production of *B. napus* increased from 37 million tons in 2003 to 73 million tons in 2014 (http://faostat3.fao.org). *B. napus* not only provides vegetable oil with superior nutritional value, its primary commercial use, but also meal for animal feed and a source of biodiesel with excellent flow properties in cold weather as a result of its low saturation.

This review summarizes current knowledge regarding drought responses of canola, with the major focus on *B. napus*. This topic is of interest from both basic and applied science viewpoints, because for most crops drought is the major abiotic stress causing severe reduction in productivity (Jensen *et al.*, 1996b; Angadi *et al.*, 2004; Willenborg *et al.*, 2004; Sinaki *et al.*, 2007). In this article we first review the physiological effects of drought on canola and then describe current knowledge in three areas relevant to modern strategies to improve drought tolerance: results from translational strategies based on discoveries made in the close relative, *A. thaliana*; large-scale datasets arising from direct -omics analyses in canola itself; and information on canola from contemporary genetic approaches such as genome-wide association studies (GWAS) and analysis of natural variation (Fig. 1). Given the tools and information available, particularly in conjunction with the recent publication of a *B. napus* genome sequence (Chalhoub *et al.*, 2014), we contend that canola is poised to become a crop model system in its own right.

II. Physiological complexity of responses to drought stress in canola crops

Investigations of physiological responses to drought in *B. napus* (Fig. 2) have been conducted under both field and growth chamber conditions (Jensen *et al.*, 1996a,b; Qaderi *et al.*, 2006; Shafiq *et al.*, 2014). Well-known processes influenced by drought stress include photosynthesis, stomatal conductance, transpiration, protein synthesis, and metabolite accumulation, all of which directly or indirectly affect seed yield and quality (Jensen *et al.*, 1996a; Hashem *et al.*, 1998; Sangtarash *et al.*, 2009).

Brassica napus is sensitive to water deficit during all stages of growth, from germination to seed set. Owing to the fact that abscisic acid (ABA) biosynthesis is induced by drought stress, ABA application is often used as a proxy for a drought signal. In *B. napus* seeds, exogenous application of ABA prevented entrance of the embryo into the growth phase (Schopfer & Plachy, 1984). ABA-mediated embryo dormancy was reported to result at least in part from a reversible inhibition of changes in cell wall biophysical

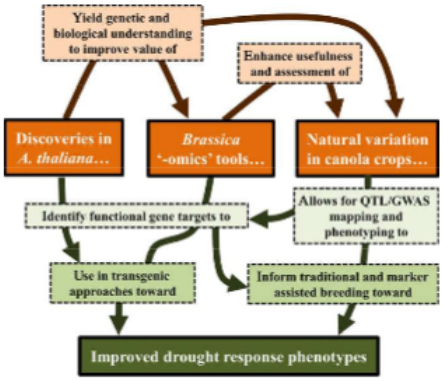

Fig. 1 Discoveries in *Arabidopsis thaliana*, *Brassica* -omics tools, and natural variation in *Brassica* species provide complementary and synergistic research approaches. When combined, these tools can identify the genetic basis for stress response traits that may yield advances in efforts to improve canola drought tolerance by transgenic and breeding strategies. QTL, quantitative trait loci; GWAS, genome-wide association studies.

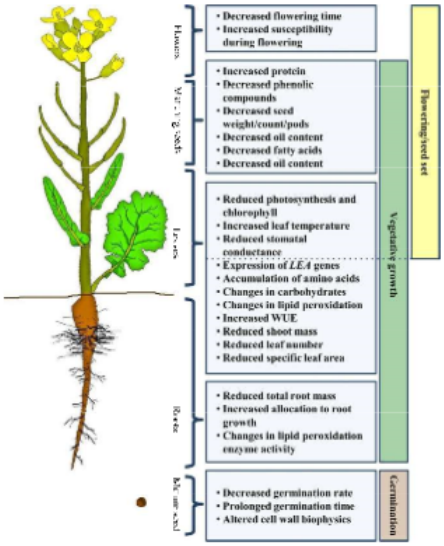

Fig. 2 Physiological and morphological trait responses to water deficit stress in canola crops discussed in this review, organized by organ type. LEA, late embryogenesis abundant; WUE, water-use efficiency.

properties, for example, cell wall extensibility coefficient and minimum turgor required for cell expansion (Schopfer & Plachy, 1985). Prolonged germination time and dramatically decreased germination rate in *B. napus* were also observed upon treatment with polyethylene glycol (PEG) (Willenborg *et al.*, 2004), which simulates the osmotic stress component of drought. Drought stress after seed germination also influences seedling growth: seedling height, fresh weight, and survival rate were negatively affected by PEG-simulated drought stress applied to 14 *B. napus* varieties after seed germination (Yang *et al.*, 2007). Therefore, drought stress during seed germination and initial growth not only impacts seed germination time and rate, but also has adverse effects on vegetative growth, and can ultimately result in yield loss in *B. napus* (Willenborg *et al.*, 2004; Li *et al.*, 2005; Yang *et al.*, 2007).

At the vegetative stage, numerous biochemical changes have been observed when *B. napus* is exposed to drought, including effects on both macromolecules and small molecules (metabolites). As in many other species, increased expression levels of late embryogenesis abundant (LEA) proteins have been observed in *B. napus* leaves under ABA, salt, cold, and osmotic stresses (Dalal *et al.*, 2009). Rapid accumulation of amino acids has been observed in *B. napus* during drought stress until rewatering (Good & Zaplachinski, 1994). Proline, which is involved in osmotic regulation (Ma *et al.*, 2003) and possibly in nitrogen-use efficiency (Albert *et al.*, 2012) under drought stress, accounts for the majority of amino acid accumulation (Good & Zaplachinski, 1994; Ma *et al.*, 2003; Din *et al.*, 2011). Previous studies revealed that proline content was increased significantly by drought stress in the *B. napus* varieties Okapi, RGS, Rainbow, and Dunkeld, suggesting production of compatible solutes as a mechanism of drought stress tolerance in this species, as is also commonplace in other species (Omidi, 2010; Ullah *et al.*, 2012). Besides proline, carbohydrate dynamics are also regulated by drought stress. For example, drought stress elevated concentrations of trehalose, glucose, fructose, and sucrose and decreased raffinose in *B. napus* var. Titan (Müller *et al.*, 2012).

Lipid peroxidation and antioxidant enzyme activities are also affected by drought stress. PEG simulation of drought treatments increased the content of malondialdehyde (MDA), a product of lipid peroxidation, and enzyme activities of superoxide dismutase, peroxidases, and catalase, in roots and shoots of several *B. napus* cultivars (Abedi & Pakniyat, 2010; Chai *et al.*, 2011; Wang *et al.*, 2011b; Mirzaee *et al.*, 2013). Liu *et al.* (2011) found that aminolevulinic acid (ALA) enhances the drought stress tolerance of *B. napus* seedlings, quantified as shoot biomass and chlorophyll (Chl) content, through enhancing the activities of specific antioxidant enzymes and inducing the expression of specific antioxidant enzyme genes.

Drought stress also causes complex whole-plant physiological and morphological responses. When water deficit occurs, the phytohormone ABA is synthesized and transported to leaf tissue, consequently activating guard cell responses that promote stomatal closure and inhibit stomatal opening to preserve plant hydration. Accumulation of ABA in leaves has been confirmed in drought-stressed *B. napus* seedlings (Qaderi *et al.*, 2006). Stomatal closure induced by exogenous application of ABA has been reported in isolated epidermal peels of *B. napus* (Zhu *et al.*, 2010). Lower stomatal conductance was observed in droughted *B. napus* plants than in well-watered plants, leading to leaf temperatures 1–2°C higher under drought (Hashem *et al.*, 1998). Drought stress decreases net CO_2 assimilation, photosynthetic rate, Chl content, and transpiration in most terrestrial plants, including *B. napus* (Hashem *et al.*, 1998; Din *et al.*, 2011; Qaderi *et al.*, 2012; Shafiq *et al.*, 2014). These responses are associated with the reduced stomatal conductance upon drought stress, which facilitates water conservation (Shaw *et al.*, 2005).

Water deficit results in decreased root and shoot biomass (Hashem *et al.*, 1998; Qaderi *et al.*, 2012; Ashraf *et al.*, 2013; Shafiq *et al.*, 2014). Although the plants are smaller overall, water deficit can increase the relative portion of the biomass allocated to roots, a strategy that is considered to be adaptive. In *B. napus*, a greater reduction in shoot mass is seen with drought at the vegetative stage than at the flowering stage (Ashraf *et al.*, 2013) and shortened shoot height can be accompanied by increased root length in drought-stressed plants (Qaderi *et al.*, 2012; Ashraf *et al.*, 2013). Drought stress also reduces leaf number and area, leaf area ratio (leaf area : plant dry weight (DW) ($cm^2\ g^{-1}$)), and transpiration, and increases water-use efficiency (WUE), and specific leaf weight (leaf DW : leaf area ($g\ m^{-2}$)) and leaf weight ratio (leaf DW : plant DW) in *B. napus* seedlings (Hashem *et al.*, 1998; Qaderi *et al.*, 2012). These growth parameters can be employed to assess the severity of drought stress.

Flowering is a critical stage influencing the yield of *B. napus*. Effects arising from drought stress imposed during vegetative growth, such as reduced net photosynthesis and stomatal conductance resulting in increased leaf temperature, were also observed in *B. napus* undergoing drought stress at flowering. Drought stress treatments imposed at flowering reduced seed weight, total seed yield, seed number per pod, and pod number per plant, and resulted in higher yield loss than drought stress applied at the vegetative stage (Champolivier & Merrien, 1996; Hashem *et al.*, 1998; Din *et al.*, 2011).

Yield in oilseed crops is positively correlated with total water availability (Nuttall *et al.*, 1992). It has been reported that after the first 6–8 inches (152–203 mm) of water, canola grain yield can increase by 150–280 kg ha^{-1} per each additional inch of water (Nielsen, 1997; Si & Walton, 2004). In Europe, for example, yields of winter canola are double those of spring varieties, and this is attributed in part to the fact that winter canola experiences minimal water deficit stress (Wan *et al.*, 2009). Drought stress imposed at the reproductive stage has a more severe impact on yield than drought stress imposed during vegetative growth, as a result of reduced pod number, seed number, and seed weight (Sinaki *et al.*, 2007; Ahmadi & Bahrani, 2009). In one experiment, plants undergoing drought stress during reproduction had *c.* 20–40% reduction in seed yield compared with nonstressed plants (Ahmadi & Bahrani, 2009).

A key agronomic issue for oilseed crops such as canola is not only the effect of drought on yield but the effect of drought on seed quality. Several studies have investigated changes in the biochemical composition of canola seeds produced under drought conditions. Drought stress at any developmental stage decreases seed oil content (Bouchereau *et al.*, 1996; Champolivier & Merrien, 1996) and alters seed oil composition (Enjalbert *et al.*, 2013). In particular, a decrease in fatty acids such as linolenic acid was observed in *B. juncea* under limited water availability (rainfed) conditions (Enjalbert *et al.*, 2013). An increase in total glucosinolate concentration was observed in *B. napus* seeds from plants undergoing drought stress during vegetative and flowering stages; however, application of water stress after flowering caused little to no change in the total glucosinolate concentration of seeds (Bouchereau *et al.*, 1996; Champolivier & Merrien, 1996; Jensen *et al.*, 1996b). Water shortages during either vegetative or flowering stages resulted in significant increases in seed protein concentration (Bouchereau *et al.*, 1996; Champolivier & Merrien, 1996; Jensen *et al.*, 1996b) and inhibited accumulation of phenolic compounds in seeds (Bouchereau *et al.*, 1996). Therefore, water shortage at any stage has potential effects on seed quality and yield in *B. napus*.

III. Translational biology: iterating between *A. thaliana* and *B. napus*

1. *Brassica* genomics and ABA signaling

While a high-density genetic linkage map of *B. napus* was generated in 2011 (Wang *et al.*, 2011a), the first complete *B. napus* genome, that of the *B. napus* European winter cv 'Darmor-*bzh*', was not reported until 2014. RNA-Seq and expressed sequence tag (EST) data in combination with *ab initio* gene prediction from the genome sequence led to the identification of *c.* 101 000 gene models, with over 90% confirmed by matching to the *B. rapa* and/or *B. oleracea* predicted proteomes (Chalhoub *et al.*, 2014). Almost half (48%) of the genes were estimated to undergo alternative splicing, mainly from intron retention. Of the assembled genome, 34.8% is composed of transposons, with their positions largely corresponding to those in the progenitor *B. rapa* and *B. oleracea* genomes.

This early Darmor-*bzh* genome both provides an invaluable resource to *B. napus* researchers and illustrates some of the problems inherent in the assembly of allopolyploid genomes. The polyploid complexity and repeat elements made it difficult to assemble the complete genome. Misassembly can result in specific problems for the downstream design and interpretation of experiments seeking to use the assembled genome to answer specific biological questions. For example, incorrect ordering of genes will introduce errors when inferring the genes involved in a process from an experiment in which quantitative trait loci (QTLs) are identified using linkage disequlibrium between genetic markers. In the future, longer sequencing read lengths will enable reads spanning more repeat regions (Clarke *et al.*, 2009; Eid *et al.*, 2009), leading to more complete and higher quality genomes.

In contrast to the nascent stage of the *B. napus* genome assembly and annotation, the reference plant *A. thaliana* provides a fully sequenced and extensively annotated genome. *A. thaliana* has been used extensively for basic discovery research in plant sciences, especially for gene function characterization. *A. thaliana* is a genetically, evolutionarily, and physiologically close relative of *B. napus* (Noh & Amasino, 1999; Byzova *et al.*, 2004; Rana *et al.*, 2004; Parkin *et al.*, 2005). The ancestral lineages diverged *c.* 16–19 million yr ago. The two species can be crossed and the nucleotide sequence conservation is in the range of 80–90% in exons and 70% in introns (Dixelius & Forsberg, 1999; the Arabidopsis Genome Initiative, 2000; Love *et al.*, 2005). Therefore, knowledge gained from the model plant species *A. thaliana* provides valuable guidance to better understand the drought responses of its close relative *B. napus* (Zhang *et al.*, 2004) and to apply translational biology approaches for development of transgenic *B. napus* with improved drought tolerance. Results from such experiments demonstrate that canola product development based on information transfer between *A. thaliana* and *B. napus* has agronomic relevance.

Abscisic acid biosynthesis can be triggered by drought stress and accumulated ABA is transported from roots to shoots and then stomata through xylem sap. Research using the model plant *A. thaliana* has provided critical insights into the core ABA signaling pathway. 'PYR/PYL/RCAR' family ABA receptors have been identified (Ma *et al.*, 2009; Park *et al.*, 2009). These receptors interact with type 2C protein phosphatases (PP2Cs), and consequently inhibit PP2Cs' function of blocking activity of downstream sucrose nonfermenting (SNF)-related kinase 2 (SnRK2) proteins, particularly OST1 (Li *et al.*, 2000; Mustilli *et al.*, 2002). After activation, OST1 phophorylation of NADPH oxidase, K^+ and anion channels, and transcription factors are central processes in ABA signal transduction (Geiger *et al.*, 2009; Sato *et al.*, 2009;

Sirichandra *et al.*, 2009, 2010). The PYR/PYL/RCAR receptors, PP2Cs, and SnRK2 form a key complex referred to as an 'ABA signalosome'. Other important components in ABA signal transduction that have been extensively studied in guard cells include reactive oxygen species (ROS) and nitric oxide production, phosphatidic acid signaling, heterotrimeric G protein-coupled signaling, and cytosolic Ca^{2+} ($[Ca^{2+}]_{cyt}$) and pH increases (for reviews on these topics, see Hubbard *et al.*, 2010 and Umezawa *et al.*, 2010).

Because the *B. napus* genome project used syntenic analysis to map *B. napus* genes to the *B. rapa* and *B. oleracea* progenitors and back to *A. thaliana* (Chalhoub *et al.*, 2014), we were able to investigate known ABA signaling pathway genes in *B. napus*. Using the ABA signaling pathway in *A. thaliana* as defined by Hauser *et al.* (Hauser *et al.*, 2011), we succeeded in finding corresponding orthologs of each *A. thaliana* ABA signaling pathway gene in *B. napus*. The distribution of the number of *B. napus* orthologs per *A. thaliana* gene indicates that the ABA pathway in *B. napus* typically retains genes from both the *B. rapa* and *B. oleracea* progenitors. As shown in Fig. 3, most Arabidopsis ABA signaling genes are represented in the *B. napus* genome as one copy from each of the two progenitors, although for a few of these ABA signaling genes *B. napus* has two or three copies from each of the ancestral genomes. There does not seem to be strong evidence for selective deletion of copies of a particular gene from one ancestor as a result of the presence of one or more copies from the other ancestor.

The lack of selective gene deletion from one or the other progenitor genomes in the ABA signalosome of Fig. 3 is perhaps expected given the recent speciation event for *B. napus*, compared with the estimated timescale for loss or mutation of gene copies (Lynch & Conery, 2000; Moore & Purugganan, 2005). Previous work in other species (Adams *et al.*, 2003; Chen, 2007) has found

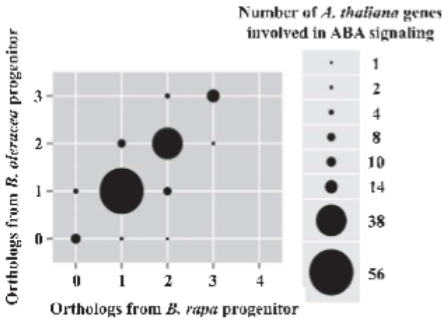

Fig. 3 Orthologs of *Arabidopsis thaliana* abscisic acid (ABA) signaling genes appear in multiple copies in the *Brassica napus* genome as a result of retention of both the *Brassica rapa* and *Brassica oleracea* progenitor genomes. *A. thaliana* ABA signaling genes were summarized by Hauser *et al.* (2011) based on the literature. The size of each circle denotes the number of Arabidopsis genes with the corresponding number of orthologs in *B. napus*, with one ortholog from each progenitor the most common, but two and three orthologs also observed.

evidence of rapid epigenetic changes, expression level differentiation, and gene silencing in polyploid plant genomes, as opposed to gene deletion. Future transcriptomic and epigenetic studies on *B. napus* should shed more light on differentiated gene expression profiles and potential silencing of genes from the A and C genomes, potentially revealing crosstalk between the *B. rapa* and *B. oleracea* drought response mechanisms present in *B. napus*. Understanding the extent of this differentiation may also suggest where polyploidy provides the potential for new and intermediate phenotypes via dosage regulation of the multiple copies present for most genes.

The above genomic analysis implicates the existence of a conserved 'ABA signalosome' in *Brassica*. This conclusion is also supported by earlier studies in which specific genes were studied. Transcription factors are important downstream targets of the ABA signaling pathway. Water stress and external ABA application up-regulate the expression of the *BolABI5* transcription factor in *B. oleracea* (Zhou *et al.*, 2013). BolABI5 is phosphorylated by BolOST1, an ortholog of AtOST1 in *B. oleracea* (Wang *et al.*, 2013). BolABI1, a *B. oleracea* ortholog of the Arabidopsis PP2C-type phosphatase, ABI1, interacts with the protein kinase BolOST1 (Wang *et al.*, 2013; Yuan *et al.*, 2013) and dephosphorylates the transcription factor BolABI5 (Yuan *et al.*, 2013). Other transcription factors have also been found to participate in ABA responses in *Brassica* species. For example, in *Arabidopsis*, AtMYC2 acts as a transcription factor involved in ABA signaling (Abe *et al.*, 1997) and the *B. napus* ortholog, *BnMYC2*, shows increased accumulation in response to drought in drought-tolerant canola lines (Aliakbari & Razi, 2013). Ying *et al.* (2014) identified a NAC domain transcription factor (*BnNAC485*) from cotyledons and young seedlings that was induced by abiotic stress and ABA treatment. *B. napus* plants overexpressing *BnNAC485* also showed hypersensitivity to exogenous ABA application (Ying *et al.*, 2014), including enhanced stomatal closing and up-regulation of ABA-responsive genes. These phenotypes were comparable to those observed in rice overexpressing the NAC transcription factor *OsSNAC1* (Hu *et al.*, 2006). Saha *et al.* (2015) recently reported that eight MADS-box transcription factors, with known function in floral organ development, were up-regulated by drought treatment in *B. rapa* seedlings (Saha *et al.*, 2015).

Signaling elements in the ABA pathway upstream of gene regulation have been particularly well studied in guard cells. Ca^{2+} elevations are a central process in guard cell ABA signaling (Hetherington *et al.*, 1986; Li *et al.*, 2006). In plants, calcineurin B-like (CBL) proteins serve as one type of calcium sensor. One family member in *A. thaliana*, CBL1, positively regulates salt and drought responses but negatively regulates cold responses (Cheong *et al.*, 2007). A variety of stresses, including salt, cold and drought, as well as ABA treatment induce the expression of another CBL family member *CBL9* in young *A. thaliana* seedlings (Pandey *et al.*, 2004). In *B. napus*, a CBL-interacting protein kinase (CIPK), *BnCIPK6*, was isolated; salt and osmotic stresses, phosphorus starvation, and ABA significantly induced the expression of both *BnCBL1* and *BnCIPK6* (Chen *et al.*, 2012). The *Arabidopsis* heterotrimeric G protein α subunit, GPA1, also has pivotal roles in multiple signaling events, including ABA-modulated stomatal movement (Wang *et al.*, 2001). The *B. napus* G protein α subunit (*BnGA1*)

gene was found to be strongly inducible by high concentrations of ABA and brassinosteroid (BR). *BnGA1* was also up-regulated by salt and drought stress but down-regulated by heat and cold stresses, indicating that G protein signaling in *B. napus*, as in *Arabidopsis*, plays important roles in both hormone signaling and environmental stress responses (Gao *et al.*, 2010). Studies such as these provide important evidence for the 'translatability' of knowledge obtained in a model species such as *A. thaliana* to its agronomically important relatives. The studies described in the next section show several successful examples of applications of such knowledge to *Brassica* crops.

2. Transgenic manipulations in *B. napus* based on knowledge derived from *A. thaliana*

Orthologs of genes identified in drought responses in *Arabidopsis* are targets for improving physiological responses to drought in *Brassica* (Zhang *et al.*, 2004). Transgenic manipulation of such genes is the most direct avenue for precise engineering of crops using discoveries from *Arabidopsis* (Table 1). In *Arabidopsis*, the β-subunit of farnesyltransferase, *ERA1*, has been shown to regulate ABA sensitivity and drought tolerance. *Arabidopsis* plants with inhibited ERA1 activity by either gene deletion or chemical inhibitor application were hypersensitive to ABA-induced anion-channel activation in guard cells and stomatal closure (Pei *et al.*, 1998). In addition, transpirational water loss is reduced in *era1* mutants upon drought treatment (Cutler *et al.*, 1996; Pei *et al.*, 1998). Wang *et al.* (2005) evaluated transgenic *B. napus* expressing an antisense *ERA1* construct driven by a drought-inducible *RD29A* promoter. Reduced germination rate and inhibited seedling development following exogenous ABA application were observed in the transgenic *B. napus* compared with nontransgenic plants. However, the transgenic plants also showed reduced stomatal conductance and enhanced ABA sensitivity under water deficit, resulting in increased seed yield under drought conditions in the field as compared with the nontransgenic wild-type plants, with no yield penalty, that is, no loss of yield under well-watered conditions (Wang *et al.*, 2005). Similarly, RNAi knockdown of the farnesyltransferase (FTA) α-subunit in *B. napus* under the shoot-specific promoter *AtHPR1* resulted in higher seed yield under drought conditions in the field than in the nontransgenic wild-type plants (Wang *et al.*, 2009). Similarly, transgenic *B. napus* lines with constitutive expression of *Arabidopsis* C-repeat/dehydration-responsive element binding factor (*CBF1*) showed enhanced drought and freezing tolerance (Jaglo *et al.*, 2001; Zhang *et al.*, 2004).

Several key enzymes in phospholipid metabolism are important components of ABA signaling pathways. For example, phosphatidic acid, a lipid-derived messenger produced by phospholipase Dα1 (PLDα1), promotes stomatal closure in *A. thaliana* (Jacob *et al.*, 1999; Zhang *et al.*, 2009). Reduced water loss and an increase in biomass accumulation and yield under stress conditions such as drought and salinity were observed in transgenic *B. napus* plants with expression of Arabidopsis *PLDα1* driven by a guard cell-specific promoter (Lu *et al.*, 2013). Another key enzyme, phosphatidylinositol-specific phospholipase C (PtdIns-PLC2), has demonstrated involvement in ABA signal transduction in *Arabidopsis* (Staxén *et al.*, 1999; Hunt *et al.*, 2003). Transgenic *B. napus* lines with constitutive overexpression of *BnPtdIns-PLC2* driven by the constitutive *CaMV35S* promoter exhibited early flowering and shorter maturation periods, accompanied by reduced transpirational rate and partially closed stomata, and enhanced drought tolerance (Georges *et al.*, 2009).

Poly (ADP-ribose) polymerase (PARP) participates in a number of cellular processes, including programmed cell death. Transgenic *B. napus* with reduced PARP activity showed reduced cell death and improved tolerance to various abiotic stresses, such as high light, drought, and high temperature (de Block *et al.*, 2005). Glycine-betaine (betaine) affords osmoprotection and protects organelles against stress conditions *in vitro*. Choline supplementation to transgenic *B. napus* with constitutive expression of a bacterial choline oxidase gene resulted in enhanced betaine accumulation. Moderate drought tolerance, assessed by measurements of relative shoot growth and net photosynthetic rate, was observed in choline-supplemented transgenic *B. napus* (Huang *et al.*, 2000).

These studies together suggest that initial elucidation of individual genes' roles in response to drought stress in a model plant species can provide fundamental knowledge to improve drought resistance in canola crops (Wan *et al.*, 2009). Commercial crop varieties arising from such *Arabidopsis*-based strategies would provide the definitive confirmation of their usefulness. As described in the next section, there are also a few examples wherein information on drought signaling and response first obtained in *B. napus* has been applied to improve drought tolerance of other species.

3. Transgenic manipulations in *A. thaliana* and other plant species based on knowledge derived from canola crops

Drought tolerance phenotypes observed in other plant species upon transgenic expression of *Brassica* genes also provide insight regarding the drought resistance function of those genes (Table 1). For example, transgenic *Arabidopsis* plants with overexpression of an active (phosphomimic) form of *B. napus* CBL-interacting protein kinase (*BnCIPK6*) showed enhanced tolerance of high-salinity and low-phosphate conditions (Chen *et al.*, 2012). These observations suggest that BnCIPK6 plays a role in responses to high salinity and phosphorus deficiency; the observation of ABA insensitivity of the Arabidopsis *cipk6* mutant also suggests a role in ABA and drought signaling (Chen *et al.*, 2012). Transgenic *Arabidopsis* plants overexpressing *B. napus* LEA gene *BnLEA4-1* under control of a constitutive *CaMV35S* or stress-inducible *RD29A* promoter both exhibited better recovery after 15 d of drought stress as compared with wild-type plants (Dalal *et al.*, 2009). Transgenic *B. campestris* overexpressing the *B. napus* group 3 *LEA* gene *BnLEA* driven by the *CaMV35S* promoter also exhibited enhanced drought tolerance, based on the survival rate after 2 wk of water deprivation, as well as improved salt tolerance as assessed from seed germination and growth performance (Park *et al.*, 2005). An ethylene-responsive factor (ERF) gene from *B. rapa*, *BrERF4*, was found to be induced by treatment with ethylene or methyl jasmonate, but not responsive to ABA or salt

Table 1 A summary of phenotypes related to drought stress in transgenic canola lines and noncanola transgenics with *Brassica* genes

Study	Promoter::Gene	GenBank ID*	Species	Transgenics	Phenotypes
Transgenic canola plants					
Huang *et al.* (2000)	*CaMV35S::COX* (choline oxidase gene from *Arthrobacter pascens*)	Not available	*B. napus*	Constitutive overexpression	Enhanced betaine accumulation; moderate drought tolerance when supplemented with choline
Jaglo *et al.* (2001); Zhang *et al.* (2004)	*CaMV35S::AtCBF1* (C-repeat/dehydration-responsive element binding factor)	NM_118681/At4 g25490	*B. napus*	Constitutive overexpression	Enhanced drought and freezing tolerance
de Block *et al.* (2005)	*CaMV35S::AtPARPs* (Poly ADP-ribose polymerase)	Z48243/At4 g02390; AJ131705/At2 g31320	*B. napus*	Constitutive overexpression	Reduced cell death and improved tolerance to various abiotic stresses, such as high light, drought, and high temperature
Wang *et al.* (2005)	*RD29A promoter* (drought inducible)::*AtERA1* (β subunit of farnesyltransferase)	BT033079/At3 g59380	*B. napus*	Antisense	Reduced germination rate and inhibited seedling development upon exogenous ABA application; reduced stomatal conductance, enhanced ABA sensitivity, and increased seed yield under drought
Georges *et al.* (2009)	*CaMV35S:: BnPtdIns-PLC2* (phosphatidylinositol-specific phospholipase C)	AF108123	*B. napus*	Constitutive overexpression	Early flowering and shorter maturation periods; reduced transpirational rate and partially closed stomata; enhanced drought tolerance
Wang *et al.* (2009)	*AtHPR1* promoter (shoot-specific)::*BnFTA* (α subunit of farnesyltransferase)	XM_013820435	*B. napus*	RNAi	Higher seed yield under drought in the field
Lu *et al.* (2013)	*AtKAT1* promoter (guard cell-specific)::*AtPLDα1* (phospholipase Dα1)	NM_112443/At3 g15730	*B. napus*	Constitutive expression	Reduced water loss, increase in biomass accumulation and yield under stress conditions such as drought and salinity
Noncanola transgenics with *Brassica* genes					
Park *et al.* (2005)	*CaMV35S:: BnLEA* (*B. napus* group 3 late embryogenesis abundant gene)	NM_001315725	*B. campestris*	Constitutive overexpression	Enhanced drought tolerance and improved salt tolerance
Yu *et al.* (2005)	*CaMV35S::BnPIP1* (*B. napus* plasma membrane aquaporin)	AF118382	*Nicotiana tabacum*	Constitutive overexpression	Reduced wilting after 10 d of water deprivation
Dalal *et al.* (2009)	*CaMV35S* or *RD29A* promoter (stress-inducible)::*BnLEA 4-1*	AY572958	*Arabidopsis thaliana*	Constitutive expression or drought-inducible	Better recovery after 15 d of drought stress
Seo *et al.* (2010)	*CaMV35S::BrERF4* (*B. rapa* ethylene-responsive factor)	XM_009137184	*A. thaliana*	Constitutive overexpression	Delayed yellowing under salt stress; greater shoot weight and a higher survival rate under drought stress
Yang *et al.* (2011)	*CaMV35S::BnLAS* (a *B. napus* ortholog of the *A. thaliana* transcriptional regulator *LAS*)	HQ324233	*A. thaliana*	Constitutive overexpression	Reduced water loss rates and enhanced drought tolerance; better recovery after dehydration
Chen *et al.* (2012)	*CaMV35S::BnCIPK6* (CBL-interacting protein kinase 6)	JF751063	*A. thaliana*	Constitutive overexpression	Enhanced high salinity and low phosphate tolerance
Chen *et al.* (2012)	*CaMV35S::BnCIPK6M* (CIPK6 phosphomimic form)	†JF751063 (T182D)	*A. thaliana*	Constitutive overexpression	Enhanced high salinity and low phosphate tolerance
Chen *et al.* (2012)	*CaMV35S::BnCIPK6*	JF751063	*A. thaliana cipk6* mutant	Constitutive overexpression	Complemented the low phosphate-sensitive and ABA-insensitive phenotypes of the mutant
Han *et al.* (2013)	*CaMV35S::BrSAC1* (a *B. rapa* phosphoinositide phosphatase)	GU434275	*N. tabacum*	Constitutive overexpression	Increased germination rate, seedling biomass, and seedling height under cold, dehydration, and salt stresses

*Both GenBank ID and AGI locus number are given for *A. thaliana* genes.
†Sequence that encodes BnCIPK6 phosphomimic form with Thr182 substituted by Asp, referred to as BnCIPK6M.

treatment in *B. rapa*. Nevertheless, overexpression of *BrERF4* in *Arabidopsis* led to delayed yellowing under salt stress as compared with the wild-type, and greater shoot weight and a higher survival rate under drought stress (Seo *et al.*, 2010). Additionally, *A. thaliana* plants with constitutive overexpression of *BnLAS*, a *B. napus* ortholog of the *A. thaliana* transcriptional regulator *LATERAL SUPPRESSOR* (*LAS*), showed reduced water loss rates and enhanced drought tolerance as well as better recovery after dehydration (Yang *et al.*, 2011).

Transgenic expression of canola genes in non-*Brassicaceous* species can also improve drought tolerance. Transgenic tobacco constitutively overexpressing the *B. napus* plasma membrane aquaporin *BnPIP1* exhibited reduced wilting after 10 d of water deprivation (Yu *et al.*, 2005). A gene encoding a phosphoinositide phosphatase from *B. rapa*, *BrSAC1*, was observed to be induced by different stress conditions, for example, cold, desiccation, salt, submergence, ABA, and heavy metals. Overexpression of *BrSAC1* in tobacco increased germination rate, seedling biomass, and seedling height under cold, dehydration, and salt stresses (Han *et al.*, 2013). All these results indicate the potential of genetic engineering at the transcriptional level for improvement of drought tolerance in crop species.

Direct modification by introduction of a protein-coding transgene (as mainly discussed earlier) is not the only strategy for genetic engineering of crops. Manipulation of gene expression towards desirable traits can also be achieved through small RNA-mediated gene silencing and epigenetic modulation, for example, DNA methylation and histone modifications. Plant microRNAs participate in a wide variety of developmental and stress (both biotic and abiotic) responses. Repression of gene expression using microRNAs has a great potential in crop improvement (please refer to Sunkar *et al.*, 2012 and Kamthan *et al.*, 2015 for reviews on this topic). Small RNAs, especially microRNAs, have been identified in canola crops through sequence-based predictions and deep sequencing (Buhtz *et al.*, 2008; Zhao *et al.*, 2012; Shen *et al.*, 2015). Some of the known canola microRNAs are development-related and stress-responsive (Pant *et al.*, 2009; Körbes *et al.*, 2012; Zhou *et al.*, 2012; Huang *et al.*, 2013; Shamloo-Dashtpagerdi *et al.*, 2015). However, at present there are relatively few canola microRNAs in the registry database (http://www.mirbase.org). *B. napus*, for example, has 90 precursors and 92 mature microRNAs, compared with Arabidopsis (325 precursors and 427 mature) or other crops (e.g. rice with 592 precursors and 713 mature). This suggests that the microRNA profile of canola crops is far from fully investigated. MicroRNAs particularly responsive to drought stress have been studied in several species, including rice (Jeong & Green, 2013), Arabidopsis (Liu *et al.*, 2008), and *Medicago truncatula* (Wang *et al.*, 2011d). The only study in canola to date identified five drought-induced microRNAs and one drought-repressed microRNA, with six transcription factors and a kinase as predicted targets (Shamloo-Dashtpagerdi *et al.*, 2015). These predicted targets are involved in ABA biosynthesis, BR and auxin signaling, and transcription (Shamloo-Dashtpagerdi *et al.*, 2015). Results from this study, together with conserved drought-responsive microRNAs discovered in other species, form an initial inventory of microRNA candidates that could potentially be manipulated to improve drought tolerance in canola. However, issues within current microRNA screening include lack of functional validation, and lack of spatial and temporal monitoring of the microRNA-induced change (Sunkar *et al.*, 2012). Therefore, investigations on tissue-specific (or even single cell type-specific) drought-responsive microRNAs along a time-course of drought treatment, together with information on expression levels of the corresponding target genes, are essential data for the goal of improved drought tolerance in canola via microRNA-based strategies.

Epigenetic features, for eample, DNA methylation and histone modifications, are associated with developmental transitions, responses to abiotic and biotic stresses, as well as numerous quantitative and qualitative traits in crops (e.g. biomass and yield; Hauben *et al.*, 2009; Verkest *et al.*, 2015). Although there is limited knowledge on the epigenome of canola as related to desirable agronomic traits (Lukens *et al.*, 2006; Gaeta *et al.*, 2007), a pioneering study showed that energy-use efficiency (EUE) is epigenetically controlled in *B. napus* (Hauben *et al.*, 2009; Verkest *et al.*, 2015). EUE was defined as the ratio of total NAD(P)H (representing the energy content) vs respiration rate (Hauben *et al.*, 2009). In general, lines with higher EUE showed global hypomethylation in genomic DNA, as well as distinct histone methylation and acetylation patterns, and these were associated with 5% yield increase (Hauben *et al.*, 2009). Furthermore, epilines (lines selected from isogenic lines, i.e. lines and varieties with identical genetic backgrounds, for traits that are epigenetically controlled) selected towards drought tolerance were generated by exposure of hypocotyl explants to 5% PEG (drought stress), and selection for low respiration was repeated over three generations. EUE was determined in the progeny of the last generation and the two epilines with highest EUE showed enhanced drought tolerance, and changes in both the transcriptome and the epigenome, particularly enrichment for regions with histone 3 lysine-4 trimethylation (H3K4me3) (Verkest *et al.*, 2015). These applications suggest significant potential for incorporating epigenetic variation into crop breeding for enhanced stress tolerance.

IV. Systems biology of *Brassica* under drought stress

Diverse physiological processes and gene categories indicate the complexity of drought responses in *B. napus*, as is also true in other species. Systems biology provides a robust tool for comprehensive understanding of drought phenotypes at different levels of biological organization. Given the rapid expansion of genomic databases and the development of -omics tools that can be applied to nonmodel species, -omics-based research on plant stress tolerance can increasingly be performed directly in the species of interest. Different fields of systems biology, for example, transcriptomics, proteomics, and metabolomics, allow simultaneous measurements of thousands of biological molecules, which generate massive datasets toward construction of a comprehensive systems picture (Hsiao & Kuo, 2006; Le Novère, 2007). Large-scale approaches have been successfully employed to understand the drought stress responses of *Brassica* species, and such transcriptomic, proteomic, and metabolomic analyses are summarized here (see Table 2 for summary).

Table 2 A summary of -omics studies on canola crops under water-deficient conditions

Study	Species/tissue	Experimental condition	Platform	Responsive biological processes
Transcriptomics				
Li *et al.* (2005)	*Brassica napus*/seed	PEG- or ABA analog PBI429- inhibited germination	Microarray	Late seed development, carbohydrate metabolism, cell wall loosening, ROS scavenging, lipolysis
Fei *et al.* (2007)	*B. napus*/seed	Natural desiccation during seed ripening stage	Microarray	Signal transductions, protein synthesis
Lee *et al.* (2008)	*B. rapa*/whole plant	Drought (air-dried)	Oligo microarray	Transcription factors
Niu *et al.* (2009)	*B. napus*/seed	Natural desiccation during seed ripening stage	cDNA chip	Fatty acid biosynthesis, auxin and jasmonate signaling
Chen *et al.* (2010)	*B. napus*/seedling root	Drought (mannitol simulation)	Macroarray	Metabolism, transcription, signal transduction, hormone and abiotic stress responses, growth and development
Bhardwaj *et al.* (2015)	*B. juncea*/seedling	Drought (mannitol simulation)	RNA-Seq	*Stress/defense responses, metabolism, phosphorylation, signal transduction, transcription and translation, cell growth, cell structure, membrane transport, circadian rhythm, catalytic activity
Shamloo-Dashtpagerdi *et al.* (2015)	*B. napus*/leaf	Drought (mannitol simulation)	Expressed sequence tag	Transcription factors, kinases, phosphatase, microRNAs
Proteomics				
Zhu *et al.* (2010)	*B. napus*/guard cells	ABA	iTRAQ	Photosynthesis, stress/defense responses, metabolism, protein synthesis, energy production, protein folding/transport and degradation, membrane transport
Mohammadi *et al.* (2012)	*B. napus*/root	Drought (irrigation control)	2D-PAGE	Metabolism, energy, disease/defense, transport
Meyer *et al.* (2012)	*B. napus*/seed	Natural desiccation during seed-ripening stage	Phosphosites mapping	Phosphorylation
Zhu *et al.* (2014)	*B. napus*/guard cells	ABA	ICAT and saturation DIGE	Thiol-based redox modification
Luo *et al.* (2015a)	*B. napus*/leaf	Short-term drought (drying on filter paper)	iTRAQ	*Ion transport, vesicle trafficking, signal perception/transduction, transcription/translation, metabolism, photosynthesis
Koh *et al.* (2015)	*B. napus*/leaf	Long-term drought (stop watering)	iTRAQ	Energy production, photosynthesis, protein synthesis, stress/defense response, metabolism, signaling, protein folding and degradation

2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; ABA, abscisic acid; DIGE, two-dimensional difference gel electrophoresis; ICAT, isotope coded affinity tag; iTRAQ, isobaric tags for relative and absolute quantitation; PEG, polyethylene glycol; ROS, reactive oxygen species. *, indicates that drought-responsive biological processes were identified by statistically significant enrichment-based on gene ontology (GO) analysis (e.g. agriGO) in the study. In other studies, biological processes were identified by representation of drought-responsive proteins/genes involved in those processes.

1. Transcriptomics

Before the availability of the genome of *B. napus* (Chalhoub *et al.*, 2014), genomes of other fully sequenced *Brassicaceae* species provided key genomic references for studies in *B. napus*. The complete genome sequence of one ancestor, *B. rapa* (var. Chiifu-401), obtained using next-generation sequencing technologies and *de novo* assembly of sequence scaffolds, was made available in 2011 (The *Brassica rapa* Genome Sequencing Project Consortium, 2011). The genome of the other ancestor, *B. oleracea*, was released in early 2014 (Liu *et al.*, 2014; sequences available at http://brassicadb.org/brad/). Additionally, the nucleotide sequence conservation between *A. thaliana* and *B. napus* allows some genomic platforms developed for *A. thaliana* also to be utilized in research on *B. napus*.

The availability of the *B. rapa* genome made microarray analysis on this species possible. A *B. rapa* oligo microarray, KBGP-24K, was constructed using sequence information from *c.* 24 000 unigenes (about half of the protein-coding genome). This array was used to analyze gene expression changes after 3-wk-old *B. rapa* plants were removed from soil and allowed to air dry in a growth chamber (Lee *et al.*, 2008). Around 3% of the genes on the microarray (738) were identified as responsive genes that were differently expressed fivefold or more at least once during the 48 h time-course of drought treatment (Lee *et al.*, 2008). This work established a useful tool to analyze *Brassica* transcripts and

highlighted a role of transcription factors during drought stress. Another study, on a *B. rapa* DH line, T12-19, used tag sequencing with a Solexa Illumina array and analyzed leaf samples under dehydration treatment for 0, 1, 2 and 3 d (Yu *et al.*, 2012). In total, 1092 genes were found to be significantly altered in response to water deficit. Among these, 37 were transcription factors, 28 were genes involved in signal transduction, and 61 were water- and osmosensing-responsive genes. The results suggested high complexity of changes at the transcriptional level under drought stress (Yu *et al.*, 2012). Taken together, such information from one of the *B. napus* ancestors provides a crucial reference toward understanding drought tolerance in *B. napus*.

The attempt to identify genome-wide drought-responsive genes in *B. napus* itself began a decade ago. Using macroarray analysis, a less expensive and less comprehensive microarray variant, a survey of genes induced by drought stresses was performed in *B. napus* (Chen *et al.*, 2010). In total, 288 clones were identified as putative drought-inducible genes, while 189 were candidates for drought-suppressed genes. These drought-responsive genes belonged to gene families participating in metabolism, transcription, signal transduction, hormone (ABA, in particular) and abiotic stress responses, as well as other processes related to growth and development (Chen *et al.*, 2010). This work, although limited owing to the methods available at the time, provided an initial gene list toward understanding drought response in *B. napus* at the transcriptional level. A recent, commercially available *B. napus* 300K microarray designed from 80 696 unigenes clustered from 543 448 ESTs and 780 cDNA provides an opportunity to substantially enhance our knowledge of stress responses in this important economic crop (Roh *et al.*, 2012), but has not yet been used in analyses of *B. napus* transcriptomic responses to drought.

As mentioned earlier, sequence similarity between *B. napus* and *A. thaliana* has allowed the use of *Arabidopsis* microarrays to profile gene expression in *Brassica*, with the caveat that paralogs may cross-hybridize and confound relative expression analyses. For example, *Arabidopsis* AR12K cDNA microarrays have been used to profile *B. napus* seed transcriptomes. In a comparison of transcriptional responses of imbibed vs germination-inhibited seeds of *B. napus*, 40 genes, mainly associated with late seed development, were up-regulated in desiccated nongerminating seeds as compared with imbibed seeds (Li *et al.*, 2005). On the other hand, 36 genes were down-regulated; these transcripts encoded proteins involved in carbohydrate metabolism, cell wall-loosening processes, ROS scavenging, and lipolysis (Li *et al.*, 2005). Specifically, the transcription factor *ABA INSENSITIVE 5* was consistently up-regulated in desiccated seeds and the gibberellic acid (GA)-induced transcription factor *PICKLE* was down-regulated. These results implicated ABA and GA signaling in the regulation of seed desiccation (Li *et al.*, 2005), and application of GA_3 (300 mg l^{-1}) was found to enhance both seed germination and seedling tolerance to drought stress in *B. napus* (Li *et al.*, 2010). Another study using the *Arabidopsis* AR12K cDNA microarrays discovered differentially expressed genes across the full-size embryo, desiccation, and mature stages of seed development in two *B. napus* cultivars (AC Excel and DH12075). Genes associated with signal transductions and protein synthesis were responsive during the desiccation stage (Fei *et al.*, 2007). In another study, a cDNA chip was generated with over 8000 EST clones from *B. napus* embryos at different stages of seed development (Niu *et al.*, 2009). Using this chip, fatty acid biosynthesis genes were found to be highly expressed in *B. napus* seeds primarily at 21 d after flowering, when seed desiccation starts. Additionally, several auxin- and jasmonate-related genes showed patterns similar to those of the fatty acid synthesis genes. Analysis of *A. thaliana* auxin and jasmonate signaling mutants revealed changes in the fatty acid components of mature seeds, indicating a link between hormone signaling, fatty acid metabolism, and desiccation (Niu *et al.*, 2009). Although desiccation is a normal component of seed development, desiccation tolerance of seeds and drought tolerance of whole plants may share some common mechanisms, because both types of stresses cause cellular dehydration (Nedeva & Nikolova, 1997).

RNA-Seq, another widely used method for genome-wide quantification of gene expression, has also been applied to identify drought-responsive genes in canola. A recent study investigated drought-responsive genes in *B. juncea* seedlings and observed that 132 transcription factors (40 induced and 92 repressed) and 452 kinases (42 induced and 410 repressed) were regulated by drought (Bhardwaj *et al.*, 2015). A similar observation was reported in an analysis of ESTs of *B. napus* under drought treatment (Shamloo-Dashtpagerdi *et al.*, 2015). This study found that 17 transcription factors, eight protein kinases, and one protein phosphatase were drought-regulated, including homologs of Arabidopsis protein phosphatase 2C *ABI1* and the ABA biosynthesis gene *ABA1*.

Although discovery of drought/desiccation-responsive genes at whole-plant and whole-organ levels provides an overall picture, studies on single cell types can provide insights into unique or cell-specific functions. In *A. thaliana*, several guard cell transcriptomic studies have been carried out. An early microarray study covering around one-third of the genome discovered 69 ABA-inducible genes and 64 ABA-repressed genes specifically in *Arabidopsis* guard cell protoplasts. Transcripts related to drought tolerance and potassium channels were among these ABA-responsive genes (Leonhardt *et al.*, 2004). Later, studies analyzing global transcriptomic responses showed a large number of ABA-regulated genes (Yang *et al.*, 2008; Wang *et al.*, 2011c; Bauer *et al.*, 2013) . An analysis was conducted using enriched preparations of *Arabidopsis* guard cells and revealed 696 ABA-induced and 477 repressed genes in this cell type (Wang *et al.*, 2011c). This study also uncovered *c.* 300 genes showing ABA regulation unique to guard cells. Collectively, these transcriptomics studies facilitate understanding of the molecular mechanisms of *Brassicaceous* species in response to drought stresses.

2. Proteomics

While transcriptome analyses constitute a facile approach for candidate gene identification, transcript abundance only indicates a putative functionality of the encoded protein and often does not reflect changes in protein abundance (Boggess *et al.*, 2013). As the final direct macromolecular product of global gene expression, analysis of the proteome is required for a thorough understanding of the cellular processes associated with drought. Early proteomic

analyses were limited both by the wet bench technologies available and by incomplete databases. Proteomics has since developed into a sophisticated research approach (Chen & Harmon, 2006). In general, comparative proteomics approaches include gel-based methods, for example, two-dimensional (2D) difference gel electrophoresis and more recent gel-free methods, for example, isobaric tags for relative and absolute quantitation (iTRAQ). Isotope multiplex labeling strategies such as iTRAQ have become popular because they overcome the limitations of gel-based proteomics methods, for example, poor resolution of membrane proteins and of very acidic or basic proteins (Chen & Harmon, 2006). Gel-based and gel-free proteomics methods complement each other and their combined use can enhance proteome coverage and identify proteins with abundance changes.

Drought-induced changes in protein patterns of *B. napus* var. *oleifera* roots were observed more than two decades ago, which might represent the earliest proteomics analysis of drought-stressed *B. napus* tissue. In the tap roots, 13 2D protein spots with low molecular weight were induced by drought. Twelve of these spots were also present in the short tuberized roots, a specific drought-induced root type. After 3 d of rehydration, the disappearance of these spots suggested their potential roles in drought tolerance (Vartanian *et al.*, 1987). However, the identities of these spots remained unknown. In a more recent study, 2D polyacrylamide gel electrophoresis was employed to investigate the initial response of *B. napus* roots to drought stress (Mohammadi *et al.*, 2012). Protein expression profiles of drought-sensitive (RGS-003) and drought-tolerant lines (SLM-003), and their F_1 hybrid, were analyzed. In the sensitive line, proteins related to metabolism, energy, disease/defense, and transport were decreased under drought stress. In the tolerant line, however, proteins involved in metabolism, disease/defense, and transport were increased, while energy-related proteins were decreased. The identified proteins with abundance changes in these lines suggest that V-type H^+-ATPase, plasma membrane-associated cation-binding protein, heat shock protein 90, and elongation factor EF-2 have a role in the drought tolerance of *B. napus*. Additionally, decreased levels of heat shock protein 70 and tubulin beta-2 in the drought-sensitive and hybrid F_1 lines might be involved in the reduced growth of these lines in drought conditions (Mohammadi *et al.*, 2012). In a recent proteomics analysis using iTRAQ, proteins responsive to short-term drought stress and salt stress were identified in leaves from 15-d-old *B. napus* seedlings. Within the proteome profile of 5583 proteins, 205 proteins showed expression level changes in response to 4 h of PEG-simulated drought treatment, with 45 common to salt-responsive proteins and 160 specific to the drought stress (Luo *et al.*, 2015a). Functional classification of the drought-responsive proteins suggested that ion transport, vesicle trafficking, and signal perception/transduction (e.g. G-protein related signaling and phosphorylation events) play a role in early drought response in *B. napus* seedlings. Additionally, notable drought-associated changes in proteins involved in transcription, translation, metabolism, and photosynthesis were observed, suggesting drought-regulation of these processes (Luo *et al.*, 2015a). In another study, the proteome response of *B. napus* leaves was studied using iTRAQ over a prolonged time-course of drought (Koh *et al.*, 2015). Respectively, 136, 244, 286, and 213 proteins were significantly altered on the 3rd, 7th, 10th, and 14th days of drought. Drought-induced proteins in *B. napus* leaves were involved in energy production, protein synthesis, and stress and defense responses, whereas drought-repressed proteins were associated with metabolism, signaling, protein folding and degradation (Koh *et al.*, 2015).

Proteomic studies have been conducted not only in *B. napus* using drought-stressed whole plants or organs but also in cell types with specialized roles in drought response. Guard cell protoplasts with high purity can be prepared on a large scale from *B. napus* leaves (Zhu *et al.*, 2009). A total of 431 nonredundant proteins were identified and quantified from untreated and ABA-treated *B. napus* guard cell protoplasts in a comparative proteomics study using iTRAQ (Zhu *et al.*, 2010). ABA up-regulated 66 proteins in *B. napus* guard cells, the majority of which were involved in photosynthesis, stress/defense responses, and metabolism. Proteins involved in photosynthesis and stress/defense responses were also observed to be drought-inducible in *B. napus* leaves (Koh *et al.*, 2015). ABA suppressed 38 proteins in *B. napus* guard cells, particularly in the categories of metabolism, protein synthesis, energy production, protein folding/transport and degradation, and membrane transport (Zhu *et al.*, 2010). The identified ABA-responsive proteins in *B. napus* guard cell protoplasts not only provide molecular details related to known physiological events in the ABA signaling pathway, for example, ROS homeostasis and cytoskeleton reorganization, but also reveal novel components in ABA signal transduction. For example, it is noteworthy that the *Arabidopsis* homolog of an ABA-induced protein, Bet v I allergen family protein, was later identified to be the ABA receptor PYL2 (Melcher *et al.*, 2009).

Proteomics approaches have been developed to identify not only those proteins that change in abundance but also proteins with changes in posttranslational modifications (PTMs), such as phosphorylation, oxidation, and glycosylation (Mann & Jensen, 2003). Posttranslational modifications of proteins are another important component of plant drought responses (Umezawa *et al.*, 2013). For example, the ABA signaling pathway is activated by initial dephosphorylation/phosphorylation events (Hubbard *et al.*, 2010). Enhanced ROS production in different cellular compartments is one of the invariant responses to drought stress (Cruz de Carvalho, 2008), which could potentially change the cellular redox status and result in protein oxidation/reduction (Martínez-Acedo *et al.*, 2012). Zhu and colleagues recently reported 65 redox-responsive proteins from *B. napus* guard cells treated with ABA. Particularly, the *in vitro* activities of an SnRK2 and a 3-isopropylmalate dehydrogenase were confirmed to be regulated by oxidant and reductant treatment (Zhu *et al.*, 2014). This study revealed thiol-based redox modification of proteins as an important regulatory mechanism in guard cell ABA signaling pathways (Zhu *et al.*, 2014). Using iTRAQ methodology, Koh and colleagues observed dynamic changes of protein PTMs (oxidation mostly, and phosphorylation) in *B. napus* leaves during drought stress (Koh *et al.*, 2015).

In a study by Meyer *et al.* (2012), over 400 phosphopeptides were identified within *B. napus* seeds at the late maturation stage. A large fraction (26.0%) of the late maturation unique

phosphopeptides were from proteins annotated as LEA proteins, which are known to play a role in dehydration tolerance (Hundertmark & Hincha, 2008). Another fraction (4.2%) was mapped to other desiccation-related proteins. Accordingly, this work supports a relationship between drought stress and seed desiccation and implicates a regulatory role of phosphorylation in these physiological processes (Meyer *et al.*, 2012).

The recent completion and publication of the *B. napus* genome sequence and anticipated progress in improved gene annotation will also provide an up-to-date database for the predicted proteome, which will allow more accurate identification of proteins in large-scale proteomics datasets generated from this species. Computational and experimentally derived proteomes can then be mined toward elucidating complex networks of protein–protein interactions. For example, protein interactions in *B. rapa* have been inferred using known *A. thaliana* interactions and interspecies homology and synteny (Yang *et al.*, 2012). A number of other methods are also available to infer interactions and regulatory networks using interaction, protein domain, and expression pattern data from related species (Liu *et al.*, 2005; Noor *et al.*, 2013). Such methods, along with availability of an expanded *A. thaliana* protein–protein interaction network (Jones *et al.*, 2014), hold promise for inferring the protein interactome of *B. napus*.

3. Metabolomics

Metabolites are also key components and regulators of biological processes. For example, stomatal closure is induced by extracellular malate and fumarate at millimolar concentrations in tomato (Araújo *et al.*, 2011). Metabolomics has emerged as a high-throughput analytical method to identify pivotal metabolites in biological processes. At present, information on global profiling of metabolites in *B. napus* is lacking, as is also true for most plant species. Two decades ago, however, evidence suggested that accumulation of free amino acids, including proline, alanine, and aspartate, is a direct effect of drought stress in *B. napus* (Good & Zaplachinski, 1994). This might be the earliest identification of key metabolites in *B. napus* drought response. Under drought conditions, considerable changes in chloroplast lipid metabolism were also observed in *B. napus* leaves. Drought stress evoked a decline in leaf polar lipids, mainly as a result of a decrease in monogalactosyldiacylglycerol content (Benhassaine-Kesri *et al.*, 2002). Furthermore, photosynthetic pigments were significantly reduced by drought stress, including Chl*a*, Chl*b*, and carotenoids in two *B. napus* varieties: Rainbow, and Dunkeld (Ullah *et al.*, 2012).

Phytohormones also participate in the regulation of drought stress response. Induction of endogenous ABA synthesis is a universal response to drought in vascular plants, including *B. napus* (Qaderi *et al.*, 2006; Wan *et al.*, 2009). In addition, the application of salicylic acid (10 μM) can ameliorate some of the adverse effects of drought stress in *B. napus*. After salicylic acid treatment, the relative water content, Chl*a* and *b*, leaf carotenoids, soluble protein, and seed oil contents recovered in drought-stressed plants to values comparable to those in well-watered plants (Ullah *et al.*, 2012). Such observations reveal a role of plant hormone crosstalk in drought stress tolerance in *B. napus*, as expected from observations on other species.

Improvements in analytical mass spectrometry (MS) have been crucial to the expansion of metabolomics. Not only the mass : charge ratio but also fragmentation information can be provided to aid in deciphering the structure of each metabolite (Dettmer *et al.*, 2007). The coupling of gas chromatography or liquid chromatography with MS allows one to profile (i.e. untargeted metabolomics) or selectively monitor (i.e. targeted metabolomics) many hundreds of compounds within a single injection (Patti *et al.*, 2012). The ionome, defined as the quantified mineral nutrients and trace elements in an organism, can be thought of as the inorganic component of the metabolome (Salt *et al.*, 2008). It is worthwhile performing high-throughput metabolomics/ionomics analysis in drought-stressed canola plants to reveal metabolome/ionome profiles of canola species and associated metabolic and nutrient networks in drought response and tolerance.

Mathematical modeling that incorporates parameters from wet laboratory measurements of metabolites and related enzymatic equations is an emerging approach to quantify and predict complicated metabolic processes at the systems level in plants (Libourel & Shachar-Hill, 2008). Among the modeling approaches, flux balance analysis (FBA) is a constraint-based method aiming to determine the mass balance by optimizing a set of flux values towards an objective function such as maximization of growth (Grafahrend-Belau *et al.*, 2009). FBA of cellular metabolism in *B. napus* has been used to predict the pathways involved in biomass accumulation under different physiological conditions of light and nutrient availability (Hay & Schwender, 2011; Pilalis *et al.*, 2011). A study in rice used FBA to model metabolic changes under drought and flooding (Lakshmanan *et al.*, 2013) and the *B. napus* metabolic model could be adjusted similarly to predict the pathways affected by drought in this species.

4. Phenomics

With advances in sequencing technologies, genomics approaches have generated massive amounts of data on gene sequences and transcriptome profiles in a great number of plant species, which provide directions for crop improvement. However, genomics alone cannot solve all the challenges in developing varieties with desirable traits, as connections between genotype and phenotype, including physiological, morphological, and phenological traits, can be indirect and highly complex. Moreover, even with identical genetic background, interaction with environmental factors results in diversity in phenotypic traits due to gene–environment interactions and the inherent plasticity of plants. Additionally, the plant phenome is itself multidimensional with numerous components, including but not limited to leaf morphology, root architecture, growth parameters, biomass, photosynthetic rate, and other physiological traits related to yield and biotic/abiotic stress responses (Furbank & Tester, 2011). Screening for favorable agronomic traits together with further understanding their underlying genetic basis may be the most promising and efficient avenue to determine gene or QTL candidates for crop improvement.

Phenotyping was manual, time-consuming, and destructive before the emergence of phenomics. Phenomics aims to use automated and reliable platforms for phenotyping in a high-throughput manner and provide traceable and reproducible data. However, owing to the complexity of the phenome, phenomics is currently limited by the availability of methods to measure certain traits. Therefore, advances in phenomics have not yet achieved the capabilities available with genomics techniques. In cereals, infrared thermography has been utilized to quantify responses in different genotypes under drought stress (Munns *et al.*, 2010), but this has yet to be applied to canola species. In canola species, an economic and high-resolution scanner system was developed to quantify root architectural traits in *B. rapa* (Adu *et al.*, 2014). Root phenomics was also reported in *B. napus* with phosphate limitation and the associated genetic loci were identified (Shi *et al.*, 2013). Similarly to Shi *et al.* (2013), phenomics in traits that are related to drought response/tolerance, for example, root elongation and biomass accumulation, could be performed in canola, and QTLs could be identified. Outcomes from such studies, together with genetic understanding, will facilitate marker-assisted selection for enhanced drought tolerance in canola.

The application of -omics and system biology approaches have already provided, and will continue to provide, in-depth knowledge of *B. napus* drought responses. Evidence for regulation of transcription, signaling pathways, protein synthesis, and metabolism, together with other processes, indicate the complexity of drought responses in *B. napus* and, presumably, most plant species. The acquired information provides potential targets for effective genetic engineering strategies towards improved stress tolerance.

V. Natural variation in drought tolerance for informing breeding

Although *B. napus* is a globally important oilseed crop, from a breeding perspective it has received relatively little attention with regard to drought responses. Drought responses as well as their underlying genetic control represent a particularly complex combination of different phenotypes. As a result, breeding strategies to date have relied largely on direct phenotypic selection for yield. There is an extensive history of using traditional mapping populations to identify QTLs for agronomic and nutritional traits in *Brassica*. Natural variation in WUE among *Brassica* lines has also been well documented (Richards, 1978; Good & Maclagan, 1993). However, despite this, improvements in drought tolerance have been limited (Cowling, 2007).

Incorporating more physiological and phenomics data in studies of drought responses may prove useful for capitalizing on the available natural variation for production of *B. napus* cultivars with improved drought tolerance. Screens of targeted aspects of drought response physiology can lead to selection of lines with altered sensitivity to drought. For example, lines exhibiting natural variation in leaf ABA sensitivity affecting stomatal water loss (Fig. 4a) may also have differences in regulation of ABA concentrations, such as catabolite content, as seen in the field (Fig. 4b). Plant types representing such stomatal dynamics vary in their response to drought conditions (Fig. 4c). Here, reduced ABA sensitivity in guard cells is correlated with decreased leaf water content under drought in the field.

In addition to physiological traits, morphological traits respond to drought stress (Fig. 2). For example, the role of root system architecture in water uptake makes it another candidate for selection. Root systems of canola crops are less dense than those of more drought-tolerant species such as wheat, and they remove less water from the soil (Cutforth *et al.*, 2013). Positive correlations between drought tolerance and increased size and depth of root systems have been found in *B. napus* (Hatzig *et al.*, 2015) and several other crop species (Cortes & Sinclair, 1986; White & Castillo, 1989; Price *et al.*, 2001; Kirkegaard & Lilley, 2007; Lopes & Reynolds, 2010). Semiautomated systems and software have been developed to characterize root architecture, which can aid in rapid phenotyping of large collections as needed for breeding (Farhidzadeh *et al.*, 2012; Galkovskyi *et al.*, 2012; Lobet & Draye, 2013; Bucksch *et al.*, 2014; Rellán-Álvarez *et al.*, 2015). However,

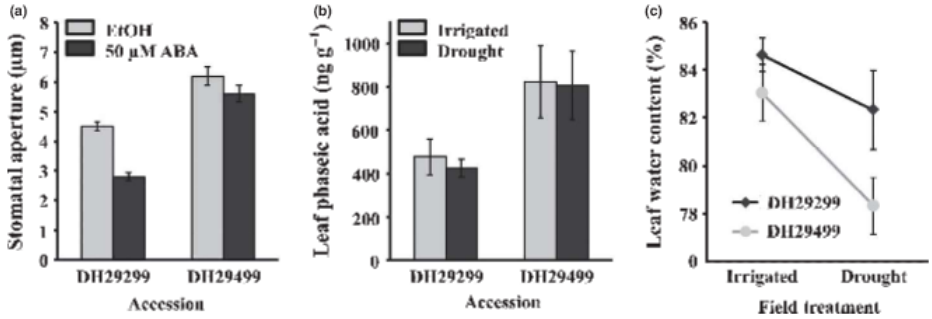

Fig. 4 Natural variation in abscisic acid (ABA)-related phenotypes in *B. napus* plants grown under laboratory and field conditions. (a) ABA sensitivity of stomatal aperture of chamber-grown plants. (b) ABA metabolism in field-grown plants. (c) Drought sensitivity of field-grown lines with different stomatal-regulation types as shown in (a). Data in (a–c) are presented as means ± standard errors.

the adaptive value of large or deep root systems depends on soil and climatic conditions, so breeding strategies need to be adjusted to match the targeted production region (Araus *et al.*, 2002; Cativelli *et al.*, 2008).

For dealing with drought stress, thus far the most common strategy in crop breeding has been to breed for drought escape, wherein plants have been selected for completing their life cycle quickly, before encountering harsh drought stress. Accordingly, current breeding practices have selected for short flowering times in *B. napus* (Rahman, 2013). As *B. napus* is most sensitive to drought during the transition from flowering to pod development (Champolivier & Merrien, 1996), this strategy is beneficial in situations of terminal drought. However, amid a changing climate and as agricultural production moves into more marginal areas and limited irrigation regimes, this strategy may prove insufficient. An alternative strategy to drought escape is dehydration avoidance, the ability to maintain internal water status upon drought stress by reducing water loss and/or enhancing water uptake. In contrast to the drought escape strategy, *B. napus* and *B. rapa* accessions with longer flowering times can have increased WUE and larger root systems for increased water uptake (Mitchell-Olds, 1996; Franks, 2011; Fletcher *et al.*, 2015). Because of the apparent tradeoff that exists between drought escape and dehydration avoidance, breeding for drought escape alone may have reduced the potential for drought tolerance among current varieties. This tradeoff has also been observed in glasshouse studies on *A. thaliana* accessions collected worldwide (McKay *et al.*, 2003; Kenney *et al.*, 2014), suggesting a widespread phenomenon. Some studies, however, have found that the negative relationship between flowering time and WUE is not invariant and there are genotypes of *A. thaliana* with both high WUE and short flowering time (Wolfe & Tonsor, 2014; Kooyers, 2015). Such genotypes with high WUE and short flowering times may exist in *Brassica* as well and could be suitable candidates for simultaneously breeding both drought tolerance strategies. Especially given the earlier mentioned tradeoff, the specific aspects of drought tolerance best suited for improvement depend on the target environment, including local details of climatic and soil moisture conditions along with irrigation practices. As the global climate warms, the co-occurrence of heat stress together with drought will further complicate this effort; for example, evaporative cooling by means of increased stomatal conductance helps to alleviate heat stress, but exacerbates drought stress.

As with many plant species, single nucleotide polymorphism (SNP) discovery in *Brassica* based on next-generation sequencing has improved the prospects for identifying natural variants of interest. Recent GWAS have identified *B. napus* variants associated with desirable agronomic traits such as seed yield and harvest index (seed biomass/vegetative biomass) (Cai *et al.*, 2014; Li *et al.*, 2014; Luo *et al.*, 2015b). These analyses have yet to be extended to drought studies under field conditions. However, a recent report by Yong *et al.* (2015) used GWAS to identify a gene controlling variation in salt tolerance in *B. napus*. This study stands as a model for the power of combing *A. thaliana* biology, *Brassica* -omics data, and natural variation toward crop improvement. Here the authors measured salt tolerance in 85 diverse inbred genotypes of *B. napus* under salinity stress. Then, using the version 4 *B. napus* genome pseudomolecules (Harper *et al.*, 2012) as a guide, they identified a set of 24 834 SNP markers in this population. A subsequent GWAS for salt tolerance revealed several QTLs. Finally, they chose candidate genes under those QTLs based on gene ontology of *A. thaliana* orthologs, and upon sequencing those genes in the *B. napus* genotypes, they identified polymorphisms in a *TSN1* (RNA-binding protein Tudor-SN) ortholog as highly explanatory of variation in salt tolerance of *B. napus*. *TSN1* is therefore a promising target for transgenic or traditional breeding for improved salt tolerance in *B. napus*. These results demonstrate the efficacy of exploring natural variation, in concert with the use of -omics and *A. thaliana* tools toward improving abiotic stress tolerance in *Brassica* crops (Fig. 1).

Genetic diversity is necessary for successful breeding of desirable traits. A number of groups have measured genetic diversity in *B. napus* (Batley *et al.*, 2003; Delourme *et al.*, 2013) and the C genome appears to have lower genetic diversity than the A genome (Wu *et al.*, 2014). There also appears to have been a loss of overall genetic diversity within at least some breeding pools, such as those in Australia (Cowling, 2007) and Canada (Fu & Gugel, 2010). Furthermore, the genetic diversity available for selective breeding within *B. napus* does not fully represent that of its parental species (Becker *et al.*, 1995; Seyis *et al.*, 2003). Therefore, in addition to the diversity within *B. napus*, the larger phenotypic diversity of other *Brassica* species could also be a source of favorable drought-related phenotypes, such as increased osmotic adjustment (Gunasekera *et al.*, 2009). Accordingly, there have been attempts to increase genetic diversity by resynthesizing *B. napus* from *B. rapa* and *B. oleracea* (Bennett *et al.*, 2012; Wu *et al.*, 2014). Additionally, there has been increased interest in introgressing loci controlling phenotypic variation using hybrid bridges and the generation of new type *B. napus*, wherein the entire A or C genome is replaced by a wild *B. rapa* or *B. oleracea* genome (Qian *et al.*, 2006; Chen *et al.*, 2011; Mei *et al.*, 2011). Indeed, Mei *et al.* (2015) demonstrated that the hybrid bridge approach successfully transferred a pathogen resistance QTL from wild *B. oleracea* into *B. napus*. This may be a powerful approach if applied to introgressing drought tolerance traits into canola crops by tapping into the vast diversity in drought responses of different wild and cultivated *Brassica* species (Richards & Thurling, 1978a,b; Kumar & Singh, 1998; Enjalbert *et al.*, 2013). Therefore, it appears that, together, the diversity of the *B. napus* gene pool and those of close relatives provide a promising resource for future selective breeding toward favorable drought tolerance traits in canola crops. The challenge will be to select for domestication traits and adaptation to agronomic management, without imposing the strong bottleneck that occurred in the original breeding of *B. napus*. Emerging methods in field-based phenomics (Andrade-Sanchez *et al.*, 2013) might allow breeding programs to work with much larger populations, and minimize the effect of drift and fixation of deleterious mutations.

VI. Conclusions/hurdles/perspectives

The availability of the *B. napus* genome has opened the door to computational as well as reverse genetic approaches that can inform

strategies to improve drought tolerance, such as analysis of promoter motifs of drought-regulated genes, studies documenting effects of copy number variation on drought tolerance, and target prediction for stress-regulated microRNAs (Xie *et al.*, 2007). Such studies were not possible with the limited genomic resources previously available in this species. The more complete picture of gene models in *B. napus* also provides an opportunity for cross-species inference of drought-regulated protein interactomes (Yang *et al.*, 2012). Knowledge of the mechanisms of drought response and resistance and their participating genes in other well-studied model species such as *A. thaliana* or crop species such as rice, maize, wheat and soybean can be used to infer parallel mechanisms in canola crops, especially when orthologous gene models are present in the canola species. Conversely, as the availability and quality of genome sequences and gene models of canola species improve, identification and subsequent manipulation of potential canola-specific genes and stress tolerance mechanisms can be accelerated.

In parallel with the genomics breakthrough in canola crops, linking phenome and genome has become urgent and indispensable to discover genes and traits contributing to canola drought tolerance. Genes related to drought tolerance in canola have been discussed earlier. Favorable traits for enhanced drought tolerance include but are not limited to: traits to enhance the plant's ability to obtain water, such as rooting depth, root architecture, water extraction capability, and ability to withstand deleterious (pathogenic) aspects, and capitalize on favorable aspects, of the microbiome; traits for improved water conservation under drought conditions, including osmoprotectant accumulation and optimized control of guard cell density, drought/ABA sensitivity and stomatal response kinetics; and traits that allow optimal plasticity in flowering time in response to varying water availability (Mullet, 2009; Ashraf, 2010).

Progress in phenomics and genomics, together with outcomes from other systems biology studies, as well as knowledge gained from other species, will deepen understanding of the mechanisms involved in drought response, adaptation, and tolerance, forming the basis and direction for canola improvement through traditional breeding or genetic engineering. Nowadays, genome manipulation is not limited to overexpressing a gene or repressing a gene through RNA interference technology (Table 1). Epigenetic modifications and the CRISPR/Cas9 system for targeted genome editing can also be applied in genetic engineering of canola crops (Belhaj *et al.*, 2015). Because *B. napus* is allopolyploid, homeologs within *B. napus* (homologs from *B. rapa* and *B. oleracea*) can share high sequence identity. CRISPR/Cas9 genome editing has been successfully applied to target two loci simultaneously in the Arabidopsis genome (Mao *et al.*, 2013). Therefore, this system has great potential for editing multiple homeologs in the *B. napus* genome. Additionally, doubled haploid (DH) lines as potential canola varieties have been developed to reduce genetic complexity and shorten breeding time for this crop (Kučera *et al.*, 2002). In combination with the earier-mentioned genetic modification strategies, obtaining DH lines (varieties) with enhanced drought tolerance can be accelerated.

It has been argued that, particularly for the phenomenon of drought tolerance, the number of successful examples wherein translation of knowledge from laboratory studies (primarily on *A. thaliana*) has resulted in adoption of a new transgenic crop cultivar, relative to the total number of studies on drought tolerance in, for example, *A. thaliana*, is disproportionately small (Passioura, 2007; Blum, 2014). Reasons that have been proffered for the low success rate include the complex, polygenic nature of plant water relations, imposition of unrealistic drought scenarios in *A. thaliana* growth chamber and glasshouse experiments, and the need to identify transgenes that will result in optimal plant performance in nonstressed as well as stressed field conditions, that is, the need to avoid yield drag (Blum, 2014 and reference therein). While these arguments have validity, it should also be noted that when the crop to be manipulated is more closely related to *A. thaliana*, as is the case for *B. napus*, the success rate is likely to be proportionately much higher. In addition, the value of the model plant *A. thaliana* as a reference genome cannot be overstated, as perfectly exemplified by its use in the initial annotation of the *B. napus* genome (Chalhoub *et al.*, 2014).

Nevertheless, drought adaptation is highly polygenic and new large-scale approaches that can be conducted directly in the crop species of interest, including both -omics analyses and large-scale genetic studies of natural variation and genome-wide association, signal a new era in drought research, with great potential for implementation via targeted molecular breeding. As illustrated in this review, current development of -omics and genetic tools and datasets for *B. napus* is allowing its development as a model crop species in its own right. This knowledge is enabling direct (intraspecies) approaches to improve drought tolerance in *B. napus*, as will become increasingly necessary for all major crop species if we are to successfully combat the vagaries of climate change and provide food, fuel, and shelter for over nine billion people by 2050.

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References

Abe H, Yamaguchi-Shinozaki K, Urao T, Iwasaki T, Hosokawa D, Shinozaki K. 1997. Role of Arabidopsis MYC and MYB homologs in drought- and abscisic acid-regulated gene expression. *Plant Cell* **9**: 1859–1868.

Abedi T, Pakniyat H. 2010. Antioxidant enzyme changes in response to drought stress in ten cultivars of oilseed rape (*Brassica napus* L.). *Czech Journal of Genetics and Plant Breeding* **46**: 27–34.

Adams KL, Cronn R, Percifield R, Wendel JF. 2003. Genes duplicated by polyploidy show unequal contributions to the transcriptome and organ-specific reciprocal silencing. *Proceedings of the National Academy of Sciences, USA* **100**: 4649–4654.

Adu MO, Chatot A, Wiesel L, Bennett MJ, Broadley MR, White PJ, Dupuy LX. 2014. A scanner system for high-resolution quantification of variation in root growth dynamics of *Brassica rapa* genotypes. *Journal of Experimental Botany* **65**: 2039–2048.

Ahmadi M. 2010. Effect of zinc and nitrogen fertilizer rates on yield and yield components of oilseed rape (*Brassica napus* L.). *World Applied Sciences Journal* **10**: 298–303.

Ahmadi M, Bahrani MJ. 2009. Yield and yield components of rapeseed as influenced by water stress at different growth stages and nitrogen levels. *American-Eurasian Journal of Agricultural and Environmental Sciences* **5**: 755–761.

Albert B, Le Cahérec F, Niogret MF, Faes P, Avice JC, Leport L, Bouchereau A. 2012. Nitrogen availability impacts oilseed rape (*Brassica napus* L.) plant water status and proline production efficiency under water-limited conditions. *Planta* **236**: 659–676.

Aliakbari M, Razi H. 2013. Isolation of *Brassica napus MYC2* gene and analysis of its expression in response to water deficit stress. *Molecular Biology Research Communications* **2**: 63–71.

Al-Shehbaz I. 1984. The tribes of crucifer (*Brassicaceae*) in the south-eastern United States. *Journal of the Arnold Arboretum* **65**: 343–373.

Andrade-Sanchez P, Gore MA, Heun JT, Thorp KR, Carmo-Silva AE, French AN, Salvucci ME, White JW. 2013. Development and evaluation of a field-based high-throughput phenotyping platform. *Functional Plant Biology* **41**: 68–79.

Angadi S, Cutforth H, McConkey B, Gan Y. 2004. Yield adjustment by canola grown at different plant populations under semiarid conditions. *Crop Science* **43**: 1358–1366.

Arabidopsis Genome Initiative. 2000. Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. *Nature* **408**: 796–815.

Araújo WL, Nunes-Nesi A, Osorio S, Usadel B, Fuentes D, Nagy R, Balbo I, Lehmann M, Studart-Witkowski C, Tohge T *et al.* 2011. Antisense inhibition of the iron-sulphur subunit of succinate dehydrogenase enhances photosynthesis and growth in tomato *via* an organic acid-mediated effect on stomatal aperture. *Plant Cell* **23**: 600–627.

Araus JL, Slafer GA, Reynolds MP, Royo C. 2002. Plant breeding and drought in C_3 cereals: what should we breed for? *Annals of Botany* **89**: 925–940.

Ashraf M. 2010. Inducing drought tolerance in plants: recent advances. *Biotechnology Advances* **28**: 169–183.

Ashraf M, Shahbaz M, Ali Q. 2013. Drought-induced modulation in growth and mineral nutrients in canola (*Brassica napus* L.). *Pakistan Journal of Botany* **45**: 93–98.

Batley AJ, Vecchies AA, Mogg BR, Bond BJ, Cogan NA, Hopkins CA, Gororo NC, Marcroft S, Forster J, Spangenberg G *et al.* 2003. A study of genetic diversity among *Brassica napus* and *Brassica juncea* germplasm collections using simple sequence repeat (SSR) molecular markers. *13th Australian Research Assembly on Brassicas – Conference Proceedings*. Sydney, NSW, Australia: NSW Agriculture, 84–86.

Bauer H, Ache P, Lautner S, Fromm J, Hartung W, Al-Rasheid KA, Sonnewald S, Sonnewald U, Kneitz S, Lachmann N *et al.* 2013. The stomatal response to reduced relative humidity requires guard cell-autonomous ABA synthesis. *Current Biology* **23**: 53–57.

Becker HC, Engqvist GM, Karlsson B. 1995. Comparison of rapeseed cultivars and resynthesized lines based on allozyme and RFLP markers. *Theoretical and Applied Genetics* **91**: 62–67.

Belhaj K, Chaparro-Garcia A, Kamoun S, Patron NJ, Nekrasov V. 2015. Editing plant genomes with CRISPR/Cas9. *Current Opinion in Biotechnology* **32**: 76–84.

Benhassaine-Kesri G, Aid F, Demandre C, Kader JC, Mazliak P. 2002. Drought stress affects chloroplast lipid metabolism in rape (*Brassica napus*) leaves. *Physiologia Plantarum* **115**: 221–227.

Bennett RA, Séguin-Swartz G, Rahman H. 2012. Broadening genetic diversity in canola using the C-genome species L. *Crop Science* **52**: 2030–2039.

Bhardwaj AR, Joshi G, Kukreja B, Malik V, Arora P, Pandey R, Shukla RN, Bankar KG, Katiyar-Agarwal S, Goel S *et al.* 2015. Global insights into high temperature and drought stress regulated genes by RNA-Seq in economically important oilseed crop *Brassica juncea*. *BMC Plant Biology* **15**: 9.

de Block M, Verduyn C, De Brouwer D, Cornelissen M. 2005. Poly (ADP-ribose) polymerase in plants affects energy homeostasis, cell death and stress tolerance. *Plant Journal* **41**: 95–106.

Blum A. 2014. Genomics for drought resistance – getting down to earth. *Functional Plant Biology* **41**: 1191–1198.

Boggess MV, Lippolis JD, Hurkman WJ, Fagerquist CK, Briggs SP, Gomes AV, Righetti PG, Bala K. 2013. The need for agriculture phenotyping: "moving from genotype to phenotype". *Journal of Proteomics* **93**: 20–39.

Bouchereau A, Clossais-Besnard N, Bensaoud A, Leport L, Renard M. 1996. Water stress effects on rapeseed quality. *European Journal of Agronomy* **5**: 19–30.

Boyer JS. 1982. Plant productivity and environment. *Science* **218**: 443–448.

Bucksch A, Burridge J, York LM, Das A, Nord E, Weitz JS, Lynch JP. 2014. Image-based high-throughput field phenotyping of crop roots. *Plant Physiology* **166**: 470–486.

Buhtz A, Springer F, Chappell L, Baulcombe DC, Kehr J. 2008. Identification and characterization of small RNAs from the phloem of *Brassica napus*. *Plant Journal* **53**: 739–749.

Byzova M, Verduyn C, De Brouwer D, De Block M. 2004. Transforming petals into sepaloid organs in Arabidopsis and oilseed rape: implementation of the hairpin RNA-mediated gene silencing technology in an organ-specific manner. *Planta* **218**: 379–387.

Cai D, Xiao Y, Yang W, Ye W, Wang B, Younas M, Wu J, Liu K. 2014. Association mapping of six yield-related traits in rapeseed (*Brassica napus* L.). *Theoretical and Applied Genetics* **127**: 85–96.

Cativelli L, Rizza F, Badeck FW, Mazzucotelli E, Mastrangelo AM, Francia E, Maré C, Tondelli A, Stanca AM. 2008. Drought tolerance improvement in crop plants: an integrated view from breeding to genomics. *Field Crops Research* **105**: 1–14.

Chai L, Wang J, Fan Z, Liu Z, Li X. 2011. Ascorbate peroxidase gene from *Brassica napus* enhances salt and drought tolerances in *Arabidopsis thaliana*. *African Journal of Biotechnology* **10**: 18085–18091.

Chalhoub B, Denoeud F, Liu S, Parkin IA, Tang H, Wang X, Chiquet J, Belcram H, Tong C, Samans B *et al.* 2014. Early allopolyploid evolution in the post-Neolithic *Brassica napus* oilseed genome. *Science* **345**: 950–953.

Champolivier L, Merrien A. 1996. Effects of water stress applied at different growth stages to *Brassica napus* L. var. *oleifera* on yield, yield components and seed quality. *European Journal of Agronomy* **5**: 153–160.

Chen L, Ren F, Zhong H, Feng Y, Jiang W, Li X. 2010. Identification and expression analysis of genes in response to high-salinity and drought stresses in *Brassica napus*. *Acta Biochimica et Biophysica Sinica (Shanghai)* **42**: 154–164.

Chen L, Ren F, Zhou L, Wang Q, Zhong H, Li X. 2012. The *Brassica napus* Calcineurin B-Like 1/CBL-interacting protein kinase 6 (CBL1/CIPK6) component is involved in the plant response to abiotic stress and ABA. *Journal of Experimental Botany* **63**: 6211–6222.

Chen S, Harmon AC. 2006. Advances in plant proteomics. *Proteomics* **6**: 5504–5516.

Chen S, Nelson MN, Chevre AM, Jenczewski E, Li Z, Mason AS, Meng J, Plummer JA, Pradhan A, Siddique KHM *et al.* 2011. Trigenomic bridges for *Brassica* improvement. *Plant Science* **30**: 524–547.

Chen ZJ. 2007. Genetic and epigenetic mechanisms for gene expression and phenotypic variation in plant polyploids. *Annual Review of Plant Biology* **58**: 377–406.

Cheong YH, Pandey GK, Grant JJ, Batistic O, Li L, Kim B-G, Lee S-C, Kudla J, Luan S. 2007. Two calcineurin B-like calcium sensors, interacting with protein kinase CIPK23, regulate leaf transpiration and root potassium uptake in Arabidopsis. *Plant Journal* **52**: 223–239.

Clarke J, Wu HC, Jayasinghe L, Patel A, Reid S, Bayley H. 2009. Continuous base identification for single-molecule nanopore DNA sequencing. *Nature Nanotechnology* **4**: 265–270.

Cortes PM, Sinclair TR. 1986. Water relations of field-grown soybean under drought. *Crop Science* **26**: 993–998.

Cowling WA. 2007. Genetic diversity in Australian canola and implications for crop breeding for changing future environments. *Field Crops Research* **104**: 103–111.

Cruz de Carvalho MH. 2008. Drought stress and reactive oxygen species: production, scavenging and signaling. *Plant Signaling & Behavior* **3**: 156–165.

Cutforth HW, Angadi SV, McConkey BG, Miller PR, Ulrich D, Gulden R, Volmar KM, Entz MH, Brandt SA. 2013. Comparing rooting characteristics and soil water withdrawal patterns of wheat with alternative oilseed and pulse crops grown in the semiarid Canadian prairie. *Canadian Journal of Soil Science* **93**: 147–160.

Cutler S, Ghassemian M, Bonetta D, Cooney S, McCourt P. 1996. A protein farnesyl transferase involved in abscisic acid signal transduction in *Arabidopsis*. *Science* **273**: 1239–1241.

Dalal M, Tayal D, Chinnusamy V, Bansal KC. 2009. Abiotic stress and ABA-inducible Group 4 *LEA* from *Brassica napus* plays a key role in salt and drought tolerance. *Journal of Biotechnology* **139**: 137–145.

Delourme R, Falentin C, Fomeju BF, Boillot M, Lassalle G, André I, Duarte J, Gauthier V, Lucante N, Marty A *et al.* 2013. High-density SNP-based genetic map development and linkage disequilibrium assessment in *Brassica napus* L. *BMC Genomics* **14**: 120.

Dettmer K, Aronov PA, Hammock BD. 2007. Mass spectrometry-based metabolomics. *Mass Spectrometry Reviews* **26**: 51–78.

Din J, Khan SU, Ali I, Gurmani AR. 2011. Physiological and agronomic response of canola varieties to drought stress. *Journal of Animal and Plant Sciences* **21**: 78–82.

Dixelius C, Forsberg J. 1999. Sexual transfer of *Arabidopsis* DNA to *Brassica napus*. *Plant Breeding* **118**: 565–567.

Eid J, Fehr A, Gray J, Luong K, Lyle J, Otto G, Peluso P, Rank D, Baybayan P, Bettman B *et al.* 2009. Real-time DNA sequencing from single polymerase molecules. *Science* **323**: 133–138.

Enjalbert JN, Zheng S, Johnson JJ, Mullen JL, Byrne PF, McKay JK. 2013. *Brassicaceae* germplasm diversity for agronomic and seed quality traits under drought stress. *Industrial Crops and Products* **47**: 176–185.

Farhidzadeh H, Hashemi SN, Masoudnia S, 2012. Phenotypic analysis of *Arabidopsis thaliana* root plant with improved feature extraction and combining classifiers approach. *Artificial Intelligence and Signal Processing (AISP), 2012 16th CSI International Symposium*. New York, NY, USA: IEEE, 452–457.

Fei H, Tsang E, Cutler AJ. 2007. Gene expression during seed maturation in *Brassica napus* in relation to the induction of secondary dormancy. *Genomics* **89**: 419–428.

Fletcher RS, Mullen JL, Heiliger A, McKay JK. 2015. QTL analysis of root morphology, flowering time, and yield reveals trade-offs in response to drought in *Brassica napus*. *Journal of Experimental Botany* **66**: 245–256.

Franks SJ. 2011. Plasticity and evolution in drought avoidance and escape in the annual plant *Brassica rapa*. *New Phytologist* **190**: 249–257.

Fu Y-B, Gugel RK. 2010. Genetic diversity of Canadian elite summer rape (*Brassica napus* L.) cultivars from the pre- to post-canola quality era. *Canadian Journal of Plant Science* **90**: 23–33.

Furbank RT, Tester M. 2011. Phenomics–technologies to relieve the phenotyping bottleneck. *Trends in Plant Science* **16**: 635–644.

Gaeta RT, Pires JC, Iniguez-Luy F, Leon E, Osborn TC. 2007. Genomic changes in resynthesized *Brassica napus* and their effect on gene expression and phenotype. *Plant Cell* **19**: 3403–3417.

Galkovskyi T, Mileyko Y, Bucksch A, Moore B, Symonova O, Price CA, Topp CN, Iyer-Pascuzzi AS, Zurek PR, Fang S *et al.* 2012. GiA Roots: software for the high throughput analysis of plant root system architecture. *BMC Plant Biology* **12**: 116.

Gao Y, Li T, Liu Y, Ren C, Zhao Y, Wang M. 2010. Isolation and characterization of gene encoding G protein alpha subunit protein responsive to plant hormones and abiotic stresses in *Brassica napus*. *Molecular Biology Reports* **37**: 3957–3965.

Geiger D, Scherzer S, Mumm P, Stange A, Marten I, Bauer H, Ache P, Matschi S, Liese A, Al-Rasheid KA *et al.* 2009. Activity of guard cell anion channel SLAC1 is controlled by drought-stress signaling kinase-phosphatase pair. *Proceedings of the National Academy of Sciences, USA* **106**: 21425–21430.

Georges F, Das S, Ray H, Bock C, Nokhrina K, Kolla VA, Keller W. 2009. Over-expression of *Brassica napus* phosphatidylinositol-phospholipase C2 in canola induces significant changes in gene expression and phytohormone distribution patterns, enhances drought tolerance and promotes early flowering and maturation. *Plant, Cell & Environment* **32**: 1664–1681.

Gleick PH. 1998. *The World's water 1998–1999: the biennial report of freshwater resources*. Washington, DC, USA: Island Press.

Good AG, Maclagan JL. 1993. Effects of drought stress on the water relations in Brassica species. *Canadian Journal of Plant Science* **73**: 525–529.

Good AG, Zaplachinski ST. 1994. The effects of drought stress on free amino acid accumulation and protein synthesis in *Brassica napus*. *Physiologia Plantarum* **90**: 9–14.

Grafahrend-Belau E, Schreiber F, Koschützki D, Junker BH. 2009. Flux balance analysis of barley seeds: a computational approach to study systemic properties of central metabolism. *Plant Physiology* **149**: 585–598.

Gunasekera CP, French RJ, Martin LD, Siddique KHM. 2009. Comparison of the responses of two Indian mustard (*Brassica juncea* L.) genotypes to post-flowering soil water deficit with the response of canola (*B. napus* L.) cv. Monty. *Crop & Pasture Science* **60**: 251–261.

Hall AE, Fiebig A, Preuss D. 2002. Beyond the Arabidopsis genome: opportunities for comparative genomics. *Plant Physiology* **129**: 1439–1447.

Han KH, Jung YJ, Bayarsaikhan U, Lee IH, Choi JS, Nou IS, Cho YG, Kang KK. 2013. Overexpression of *BrSAC1* encoding a phosphoinositide phosphatase isolated from Chinese cabbage (*Brassica rapa* L) improved tolerance to cold, dehydration, and salt stresses in transgenic tobacco. *African Journal of Biotechnology* **12**: 1782–1792.

Harper AL, Trick M, Higgins J, Fraser F, Clissold L, Wells R, Hattori C, Werner P, Bancroft I. 2012. Associative transcriptomics of traits in the polyploid crop species *Brassica napus*. *Nature Biotechnology* **30**: 798–802.

Hashem A, Amin Majumdar MN, Hamid A, Hossain MM. 1998. Drought stress effects on seed yield, yield attributes, growth, cell membrane stability and gas exchange of synthesized *Brassica napus* L. *Journal of Agronomy and Crop Science* **180**: 129–136.

Hatzig SV, Schiessl S, Stahl A, Snowdon RJ. 2015. Characterizing root response phenotypes by neural network analysis. *Journal of Experimental Botany* **66**: 5617–5624.

Hauben M, Haesendonckx B, Standaert E, Van Der Kelen K, Azmi A, Akpo H, Van Breusegem F, Guisez Y, Bots M, Lambert B *et al.* 2009. Energy use efficiency is characterized by an epigenetic component that can be directed through artificial selection to increase yield. *Proceedings of the National Academy of Sciences, USA* **106**: 20109–20114.

Hauser F, Waadt R, Schroeder JI. 2011. Evolution of abscisic acid synthesis and signaling mechanisms. *Current Biology* **21**: R346–R355.

Hay J, Schwender J. 2011. Computational analysis of storage synthesis in developing *Brassica napus* L. (oilseed rape) embryos: flux variability analysis in relation to ^{13}C metabolic flux analysis. *Plant Journal* **67**: 513–525.

Hetherington AM, De Silva DLR, Cox RC, Mansfield TA. 1986. Abscisic acid, calcium ions and stomatal function. In: Trewavas AJ, ed. *Molecular and cellular aspects of calcium in plant development*. New York, NY, USA: Springer US, 387–388.

Hsiao A, Kuo M. 2006. High-throughput biology in the postgenomic era. *Journal of Vascular and Interventional Radiology* **17**: 1077–1085.

Hu H, Dai M, Yao J, Xiao B, Li X, Zhang Q, Xiong L. 2006. Overexpressing a NAM, ATAF, and CUC (NAC) transcription factor enhances drought resistance and salt tolerance in rice. *Proceedings of the National Academy of Sciences, USA* **103**: 12987–12992.

Huang D, Koh C, Feurtado JA, Tsang EW, Cutler AJ. 2013. MicroRNAs and their putative targets in *Brassica napus* seed maturation. *BMC Genomics* **14**: 140.

Huang J, Hirji R, Adam L, Rozwadowski KL, Hammerlindl JK, Keller WA, Selvaraj G. 2000. Genetic engineering of glycinebetaine production toward enhancing stress tolerance in plants: metabolic limitations. *Plant Physiology* **122**: 747–756.

Hubbard KE, Nishimura N, Hitomi K, Getzoff ED, Schroeder JI. 2010. Early abscisic acid signal transduction mechanisms: newly discovered components and newly emerging questions. *Genes & Development* **24**: 1695–1708.

Hundertmark M, Hincha DK. 2008. LEA (late embryogenesis abundant) proteins and their encoding genes in *Arabidopsis thaliana*. *BMC Genomics* **9**: 118.

Hunt L, Mills LN, Pical C, Leckie CP, Aitken FL, Kopka J, Mueller-Roeber B, McAinsh MR, Hetherington AM, Gray JE. 2003. Phospholipase C is required for the control of stomatal aperture by ABA. *Plant Journal* **34**: 47–55.

Jacob T, Ritchie S, Assmann SM, Gilroy S. 1999. Abscisic acid signal transduction in guard cells is mediated by phospholipase D activity. *Proceedings of the National Academy of Sciences, USA* **96**: 12192–12197.

Jaglo KR, Kleff S, Amundsen KL, Zhang X, Haake V, Zhang JZ, Deits T, Thomashow MF. 2001. Components of the Arabidopsis C-repeat/dehydration-responsive element binding factor cold-response pathway are conserved in *Brassica napus* and other plant species. *Plant Physiology* **127**: 910–917.

Jensen C, Morgensen V, Mortensen G, Andersen M, Schjoerring J, Thage J, Koribidis J. 1996a. Leaf photosynthesis and drought adaptation in field-grown oilseed rape (*Brassica napus* L.) *Australian Journal of Plant Physiology* **23**: 631–644.

Jensen C, Mogensen V, Mortensen G, Fieldsend J, Milford G, Andersen M, Thage J. 1996b. Seed glucosinolate, oil and protein contents of field-grown rape (*Brassica napus* L). affected by soil drying and evaporative demand. *Field Crops Research* **47**: 93–105.

Jeong DH, Green PJ. 2013. The role of rice microRNAs in abiotic stress responses. *Journal of Plant Biology* **56**: 187–197.

Jones AM, Xuan Y, Xu M, Wang RS, Ho CH, Lalonde S, You CH, Sardi MI, Parsa SA, Smith-Valle E *et al.* 2014. Border control-a membrane-linked interactome of Arabidopsis. *Science* **344**: 711–716.

Kamthan A, Chaudhuri A, Kamthan M, Datta A. 2015. Small RNAs in plants: recent development and application for crop improvement. *Frontiers in Plant Science* **6**: 208.

Kenney AM, McKay JK, Richards JH, Juenger TE. 2014. Direct and indirect selection on flowering time, water-use efficiency (WUE, $\delta^{13}C$), and WUE plasticity to drought in *Arabidopsis thaliana*. *Ecology and Evolution* **23**: 4305–4321.

Kirkegaard JA, Lilley JM. 2007. Root penetration rate – a benchmark to identify soil and plant limitations to rooting depth in wheat. *Animal Production Science* **47**: 590–602.

Koh J, Chen G, Yoo M, Zhu N, Dufresne D, Erickson JE, Shao H, Chen S. 2015. Comparative proteomic analysis of *Brassica napus* in response to drought stress. *Journal of Proteome Research* **14**: 3068–3081.

Kooyers NJ. 2015. The evolution of drought escape and avoidance in natural herbaceous populations. *Plant Science* **234**: 155–162.

Körbes AP, Machado RD, Guzman F, Almerão MP, de Oliveira LFV, Loss-Morais G, Turchetto-Zolet AC, Cagliari A, dos Santos Maraschin F, Margis-Pinheiro M *et al.* 2012. Identifying conserved and novel microRNAs in developing seeds of *Brassica napus* using deep sequencing. *PLoS ONE* **7**: e50663.

Kučera V, Vyvadilová M, Klima M. 2002. Utilization of doubled haploids in winter oilseed rape. *Czech Journal of Genetics and Plant Breeding* **38**: 50–54.

Kumar A, Singh DP. 1998. Use of physiological indices as a screening technique for drought tolerance in oilseed *Brassica* species. *Annals of Botany* **81**: 413–420.

Lakshmanan M, Zhang Z, Mohanty B, Kwon J, Choi H, Nam H, Kim D, Lee D. 2013. Elucidating rice cell metabolism under flooding and drought stresses using flux-based modeling and analysis. *Plant Physiology* **162**: 2140–2150.

Le Novère N. 2007. The long journey to a systems biology of neuronal function. *BMC Systems Biology* **1**: 28.

Lee SC, Lim MH, Kim JA, Lee SI, Kim JS, Jin M, Kwon SJ, Mun JH, Kim YK, Kim HU *et al.* 2008. Transcriptome analysis in *Brassica rapa* under the abiotic stresses using *Brassica* 24K oligo microarray. *Molecules and Cells* **26**: 595–605.

Leonhardt N, Kwak JM, Robert N, Waner D, Leonhardt G, Schroeder JI. 2004. Microarray expression analyses of Arabidopsis guard cells and isolation of a recessive abscisic acid hypersensitive protein phosphatase 2C mutant. *Plant Cell* **6**: 596–615.

Li F, Chen B, Xu K, Wu J, Song W, Bancroft I, Harper AL, Trick M, Liu S, Gao G *et al.* 2014. Genome-wide association study dissects the genetic architecture of seed weight and seed quality in rapeseed (*Brassica napus* L.). *DNA Research* **21**: 355–367.

Li F, Wu X, Tsang E, Cutler A. 2005. Transcriptional profiling of imbibed *Brassica napus* seed. *Genomics* **86**: 718–730.

Li J, Want X, Watson MB, Assmann SM. 2000. Regulation of abscisic acid-induced stomatal closure and anion channels by guard cell AAPK kinase. *Science* **287**: 300–303.

Li S, Assmann SM, Albert R. 2006. Predicting essential components of signal transduction networks: a dynamic model of guard cell abscisic acid signaling. *PLoS Biology* **4**: e312.

Li Z, Lu G, Zhang X, Zou C, Cheng Y, Zheng P. 2010. Improving drought tolerance of germinating seeds by exogenous application of gibberellic acid (GA3) in rapeseed (*Brassica napus L.*). *Seed Science and Technology* **38**: 432–440.

Libourel IG, Shachar-Hill Y. 2008. Metabolic flux analysis in plants: from intelligent design to rational engineering. *Annual Review of Plant Biology* **59**: 625–650.

Liu D, Pei Z, Naeem MS, Ming D, Liu H, Khan F, Zhou W. 2011. 5-Aminolevulinic acid activates antioxidative defence system and seedling growth in *Brassica napus* L. under water-deficit stress. *Journal of Agronomy and Crop Science* **197**: 284–295.

Liu H, Tian X, Li Y, Wu C, Zheng C. 2008. Microarray-based analysis of stress-regulated microRNAs in *Arabidopsis thaliana*. *RNA* **14**: 836–843.

Liu S, Liu Y, Yang X, Tong C, Edwards D, Parkin IA, Zhao M, Ma J, Yu J, Huang S *et al.* 2014. The *Brassica oleracea* genome reveals the asymmetrical evolution of polyploid genomes. *Nature Communications* **5**: 3930.

Liu Y, Liu N, Zhao H. 2005. Inferring protein–protein interactions through high-throughput interaction data from diverse organisms. *Bioinformatics* **21**: 3279–3285.

Lobet G, Draye X. 2013. Novel scanning procedure enabling the vectorization of entire rhizotron-grown root systems. *Plant Methods* **9**: 1.

Lopes MS, Reynolds MP. 2010. Partitioning of assimilates to deeper roots is associated with cooler canopies and increased yield under drought in wheat. *Functional Plant Biology* **37**: 147–156.

Love CG, Robinson AJ, Lim GA, Hopkins CJ, Batley J, Barker G, Spangenberg GC, Edwards D. 2005. Brassica ASTRA: an integrated database for *Brassica* genomic research. *Nucleic Acids Research* **33**: D656–D659.

Lu S, Bahn SC, Qu G, Qin H, Hong Y, Xu Q, Zhou Y, Hong Y, Wang X. 2013. Increased expression of phospholipase Dα1 in guard cells decreases water loss with improved seed production under drought in *Brassica napus*. *Plant Biotechnology Journal* **11**: 380–389.

Lukens LN, Pires JC, Leon E, Vogelzang R, Oslach L, Osborn T. 2006. Patterns of sequence loss and cytosine methylation within a population of newly resynthesized *Brassica napus* allopolyploids. *Plant Physiology* **140**: 336–348.

Luo J, Tang S, Peng X, Yan X, Zeng X, Li J, Li X, Wu G. 2015a. Elucidation of cross-talk and specificity of early response mechanisms to salt and PEG-simulated drought stresses in *Brassica napus* using comparative proteomic analysis. *PLoS ONE* **10**: e0138974.

Luo X, Ma C, Yue Y, Hu K, Li Y, Duan Z, Wu M, Tu J, Shen J, Yi B *et al.* 2015b. Unravelling the complex trait of harvest index in rapeseed (*Brassica napus* L.) with association mapping. *BMC Genomics* **16**: 379.

Lynch M, Conery JS. 2000. The evolutionary fate and consequences of duplicate genes. *Science* **290**: 1151–1155.

Ma Q, Turner DW, Levy D, Cowling WA. 2003. Solute accumulation and osmotic adjustment in leaves of *Brassica* oilseeds in response to soil water deficit. *Australian Journal of Agricultural Research* **55**: 939–945.

Ma Y, Szostkiewicz I, Korte A, Moes D, Yang Y, Christmann A, Grill E. 2009. Regulators of PP2C phosphatase activity function as abscisic acid sensors. *Science* **324**: 1064–1068.

Mann M, Jensen ON. 2003. Proteomic analysis of post-translational modifications. *Nature Biotechnology* **21**: 255–261.

Mao Y, Zhang H, Xu N, Zhang B, Gao F, Zhu JK. 2013. Application of the CRISPR-Cas system for efficient genome engineering in plants. *Molecular Plant* **6**: 2008–2011.

Martínez-Acedo P, Núñez E, Gómez FJ, Moreno M, Ramos E, Izquierdo-Álvarez A, Miró-Casas E, Mesa R, Rodríguez P, Martínez-Ruiz A *et al.* 2012. A novel strategy for global analysis of the dynamic thiol redox proteome. *Molecular & Cellular Proteomics* **11**: 800–813.

McKay JK, Richards JH, Mitchell-Olds T. 2003. Genetics of drought adaptation in *Arabidopsis thaliana*: I. Pleiotropy contributes to genetic correlations among ecological traits. *Molecular Ecology* **12**: 1137–1151.

Mei J, Fu Y, Qian L, Xu X, Li J, Qian W. 2011. Effectively widening the gene pool of oilseed rape (*Brassica napus* L.) by using Chinese *B. rapa* in a virtual allopolyploid approach. *Plant Breeding* **130**: 333–337.

Mei J, Liu Y, Wei D, Wittkop B, Ding Y, Li Q, Li J, Wan H, Li Z, Ge X *et al.* 2015. Transfer of sclerotinia resistance from wild relative of *Brassica oleracea* into *Brassica napus* using a hexaploidy step. *Theoretical and Applied Genetics* **128**: 639–644.

Melcher K, Ng LM, Zhou XE, Soon FF, Xu Y, Suino-Powell KM, Park SY, Weiner JJ, Fujii H, Chinnusamy V *et al.* 2009. A gate-latch-lock mechanism for hormone signaling by abscisic acid receptors. *Nature* **462**: 602–608.

Meyer LJ, Gao J, Xu D, Thelen JJ. 2012. Phosphoproteomic analysis of seed maturation in *Arabidopsis*, rapeseed, and soybean. *Plant Physiology* **159**: 517–528.

Mirzaee M, Moieni A, Ghanati F. 2013. Effects of drought stress on the lipid peroxidation and antioxidant enzyme activities in two canola (*Brassica napus* L.) cultivars. *Journal of Agricultural Science and Technology* **15**: 593–602.

Mitchell-Olds T. 1996. Pleiotropy causes long-term genetic constraints on life-history evolution in *Brassica rapa*. *Evolution* **50**: 1849–1858.

Mohammadi PP, Moieni A, Komatsu S. 2012. Comparative proteome analysis of drought-sensitive and drought-tolerant rapeseed roots and their hybrid F_1 line under drought stress. *Amino Acids* **43**: 2137–2152.

Moore RC, Purugganan MD. 2005. The evolutionary dynamics of plant duplicate genes. *Current Opinion in Plant Biology* **8**: 122–128.

Müller T, Lentzsch P, Müller MEH. 2012. Carbohydrate dynamics in leaves of rapeseed (*Brassica napus*) under drought. *Journal of Agronomy and Crop Science* **198**: 207–217.

Mullet J. 2009. Traits and genes for plant drought tolerance. In: Kriz AL, Larkins BA, eds. *Molecular genetic approaches to maize improvement.* Heidelberg/Berlin, Germany: Springer, 55–64.

Munns R, James RA, Sirault XR, Furbank RT, Jones HG. 2010. New phenotyping methods for screening wheat and barley for beneficial responses to water deficit. *Journal of Experimental Botany* **61**: 3499–3507.

Mustilli AC, Merlot S, Vavasseur A, Fenzi F, Giraudat J. 2002. Arabidopsis OST1 protein kinase mediates the regulation of stomatal aperture by abscisic acid and acts upstream of reactive oxygen species production. *Plant Cell* **14**: 3089–3099.

Nedeva D, Nikolova A. 1997. Desiccation tolerance in developing seeds. *Bulgarian Journal of Plant Physiology* **23**: 100–103.

Nielsen DC. 1997. Water use and yield of canola under dryland conditions in the Great Plains. *Journal of Production Agriculture* **10**: 307–313.

Niu Y, Wu G, Ye R, Lin W, Shi Q, Xue L, Xu X, Li Y, Du Y, Xue H. 2009. Global analysis of gene expression profiles in *Brassica napus* developing seeds reveals a conserved lipid metabolism regulation with *Arabidopsis thaliana*. *Molecular Plant* **2**: 1107–1122.

Noh YS, Amasino RM. 1999. Regulation of developmental senescence is conserved between *Arabidopsis* and *Brassica napus*. *Plant Molecular Biology* **41**: 195–206.

Noor A, Serpedin E, Nounou M, Nounou H, Mohamed N, Chouchane L. 2013. An overview of the statistical methods used for inferring gene regulatory networks and protein–protein interaction networks. *Advances in Bioinformatics* **2013**: 953814.

Nuttall WF, Moulin AP, Townley-Smith LJ. 1992. Yield response of canola to nitrogen, phosphorus, precipitation and temperature. *Agronomy Journal* **84**: 765–768.

Omidi H. 2010. Changes of proline content and activity of antioxidative enzymes in two canola genotype under drought stress. *American Journal of Plant Physiology* **5**: 338–349.

Palmer JD, Shields C, Cohen D, Orton T. 1983. Chloroplast DNA evolution and the origin of amphidiploid *Brassica* species. *Theoretical and Applied Genetics* **65**: 181–189.

Pandey GK, Cheong YH, Kim KN, Grant JJ, Li L, Hung W, D'Angelo C, Weinl S, Kudla J, Luan S. 2004. The calcium sensor calcineurin B-like 9 modulates abscisic Acid sensitivity and biosynthesis in Arabidopsis. *Plant Cell* **16**: 1912–1924.

Pant BD, Musialak-Lange M, Nuc P, May P, Buhtz A, Kehr J, Walther D, Scheible WR. 2009. Identification of nutrient-responsive Arabidopsis and rapeseed microRNAs by comprehensive real-time polymerase chain reaction profiling and small RNA sequencing. *Plant Physiology* **150**: 1541–1555.

Park BJ, Liu Z, Kanno A, Kameya T. 2005. Genetic improvement of Chinese cabbage for salt and drought tolerance by constitutive expression of a *B. napus LEA* gene. *Plant Science* **169**: 553–558.

Park S, Fung P, Nishimura N, Jensen DR, Fujii H, Zhao Y, Lumba S, Santiago J, Rodrigues A, Chow TF *et al.* 2009. Abscisic acid inhibits type 2C protein phosphatases via the PYR/PYL family of START proteins. *Science* **324**: 1068–1071.

Parkin IA, Gulden SM, Sharpe AG, Lukens L, Trick M, Osborn TC, Lydiate DJ. 2005. Segmental structure of the *Brassica napus* genome based on comparative analysis with *Arabidopsis thaliana*. *Genetics* **171**: 765–781.

Passioura J. 2007. The drought environment: physical, biological and agricultural perspectives. *Journal of Experimental Botany* **58**: 113–117.

Paterson AH, Lan TH, Amasino RM, Osborn TC, Quiros C. 2001. *Brassica* genomics: a complement to, and early beneficiary of, the *Arabidopsis* sequence. *Genome Biology* **2**: 1011–1014.

Patti GJ, Yanes O, Siuzdak G. 2012. Innovation: metabolomics: the apogee of the omics trilogy. *Nature Reviews Molecular Cell Biology* **13**: 263–269.

Pei Z, Ghassemian M, Kwak CM, McCourt P, Schroeder JI. 1998. Role of farnesyltransferase in ABA regulation of guard cell anion channels and plant water loss. *Science* **282**: 287–290.

Pennisi E. 2008. Plant genetics: the blue revolution, drop by drop, gene by gene. *Science* **320**: 171.

Pilalis E, Chatziioannou A, Thomasset B, Kolisis F. 2011. An *in silico* compartmentalized metabolic model of *Brassica napus* enables the systemic study of regulatory aspects of plant central metabolism. *Biotechnology and Bioengineering* **108**: 1673–1682.

Price AH, Cairns JE, Horton P, Jones HG, Griffiths H. 2001. Linking drought resistance mechanisms to drought avoidance in upland rice using a QTL approach: progress and new opportunities to integrate stomatal and mesophyll responses. *Journal of Experimental Botany* **53**: 989–1004.

Qaderi M, Kurepin L, Reid D. 2006. Growth and physiological responses of canola (*Brassica napus*) to three components of global climate change: temperature, carbon dioxide and drought. *Physiologia Plantarum* **128**: 710–721.

Qaderi M, Kurepin VL, Reid MD. 2012. Effects of temperature and watering regime on growth, gas exchange and abscisic acid content of canola (*Brassica napus*) seedlings. *Environmental and Experimental Botany* **75**: 107–113.

Qian W, Meng J, Li M, Frauen M, Sass O, Noack J, Jung C. 2006. Introgression of genomic components from Chinese *Brassica rapa* contributes to widening the genetic diversity in rapeseed (*B. napus* L.), with emphasis on the evolution of Chinese rapeseed. *Theoretical and Applied Genetics* **113**: 49–54.

Rahman H. 2013. Breeding spring canola (*Brassica napus* L.) by the use of exotic germplasm. *Canadian Journal of Plant Science* **93**: 363–373.

Rana D, van den Boogaart T, O'Neill CM, Hynes L, Bent E, Macpherson L, Park JY, Lim YP, Bancroft I. 2004. Conservation of the microstructure of genome segments in *Brassica napus* and its diploid relatives. *Plant Journal* **40**: 725–733.

Rellán-Álvarez R, Lobet G, Lindner H, Pradier PLM, Yee MC, Sebastian J, Geng Y, Trontin C, LaRue T, Lavelle AS *et al.* 2015. Multidimensional mapping of root responses to soil environmental cues using a luminescence-based imaging system. *bioRxiv* 016931.

Richards RA. 1978. Genetic analysis of drought stress response in rapeseed (*Brassica campestris* and *B. napus*). I. Assessment of environments for maximum selection response in grain yield. *Euphytica* **27**: 609–615.

Richards RA, Thurling N. 1978a. Variation between and within species of rapeseed (*Brassica campestris* and B. napus) in response to drought stress. I. Sensitivity at different stages of development. *Crop & Pasture Science* **29**: 469–477.

Richards RA, Thurling N. 1978b. Variation between and within species of rapeseed (*Brassica campestris* and B. napus) in response to drought stress. II. Growth and development under natural drought stresses. *Crop & Pasture Science* **29**: 479–490.

Roh KH, Park J, Kim J, Kim HU, Lee K, Kim SH. 2012. Gene expression profiling of oilseed rape embryos using microarray analysis. *Journal of Applied Biological Chemistry* **55**: 227–234.

Rosegrant MW, Fernandez M, Sinha A, Alder J, Ahammad H, de Fraiture C, Eickhour B, Fonseca J, Huang J, Koyama O *et al.* 2009. Looking into the future for agriculture and AKST (Agricultural Knowledge Science and Technology). In: McIntyre BD, Herren HR, Wakhungu J, Watson RT, eds. *Agriculture at a crossroads*. Washington, DC, USA: Island Press, 307–376.

Saha G, Park JI, Jung HJ, Ahmed NU, Kayum A, Chung MY, Hur Y, Cho YG, Watanabe M, Nou IS. 2015. Genome-wide identification and characterization of MADS-box family genes related to organ development and stress resistance in *Brassica rapa*. *BMC Genomics* **16**: 178.

Salt DE, Baxter I, Lahner B. 2008. Ionomics and the study of the plant ionome. *Annual Review of Plant Biology* **59**: 709–733.

Sangtarash M, Qaderi M, Chinnappa C, Reid D. 2009. Differential sensitivity of canola (*Brassica napus*) seedlings to ultraviolet-B radiation, water stress and abscisic acid. *Environmental and Experimental Botany* **66**: 212–219.

Sato A, Sato Y, Fukao Y, Fujiwara M, Umezawa T, Shinozaki K, Hibi T, Taniguchi M, Miyake H, Goto DB *et al.* 2009. Threonine at position 306 of the KAT1 potassium channel is essential for channel activity and is a target site for ABA-activated SnRK2/OST1/SnRK2. 6 protein kinase. *Biochemical Journal* **424**: 439–448.

Schmidt R, Bancroft I. 2011. *Genetics and genomics of the Brassicaceae*. New York, NY, USA: Springer.

Schopfer P, Plachy C. 1984. Control of seed germination by abscisic acid: II. Effect on embryo water uptake in *Brassica napus* L. *Plant Physiology* **76**: 155–160.

Schopfer P, Plachy C. 1985. Control of seed germination by abscisic acid: III. Effect on embryo growth potential (minimum turgor pressure) and growth coefficient (cell wall extensibility) in *Brassica napus* L. *Plant Physiology* **77**: 676–686.

Seo YJ, Park JB, Cho YJ, Jung C, Seo HS, Park SK, Nahm BH, Song JT. 2010. Overexpression of the ethylene-responsive factor gene *BrERF4* from *Brassica rapa*

increases tolerance to salt and drought in Arabidopsis plants. *Molecules and Cells* **30**: 271–277.

Seyis F, Snowdon RJ, Luhs W, Friedt W. 2003. Molecular characterization of novel resynthesized rapeseed (*Brassica napus*) lines and analysis of their genetic diversity in comparison with spring rapeseed cultivars. *Plant Breeding* **122**: 473–478.

Shafiq S, Akram NA, Ashraf M, Arshad A. 2014. Synergistic effects of drought and ascorbic acid on growth, mineral nutrients and oxidative defense system in canola (*Brassica napus* L.) plants. *Acta Physiologiae Plantarum* **36**: 1539–1553.

Shamloo-Dashtpagerdi R, Razi H, Ebrahimie E. 2015. Mining expressed sequence tags of rapeseed (*Brassica napus* L.) to predict the drought responsive regulatory network. *Physiology and Molecular Biology of Plants* **21**: 329–340.

Shaw MR, Huxman TE, Lund CP. 2005. Modern and future semi-arid and arid ecosystems. In: Ehleringer JR, Cerling TE, Dearing MD, eds. *A history of atmospheric CO2 and its effects on plants, animals, and ecosystems.* New York, NY, USA: Springer, 415–440.

Shen E, Zou J, Behrens FH, Chen L, Ye C, Dai S, Li R, Ni M, Jiang X, Qiu J *et al.* 2015. Identification, evolution, and expression partitioning of miRNAs in allopolyploid *Brassica napus*. *Journal of Experimental Botany* **66**: 7241–7253.

Shi L, Shi T, Broadley MR, White PJ, Long Y, Meng J, Xu F, Hammond JP. 2013. High-throughput root phenotyping screens identify genetic loci associated with root architectural traits in *Brassica napus* under contrasting phosphate availabilities. *Annals of Botany* **112**: 381–389.

Si P, Walton GH. 2004. Determinants of oil concentration and seed yield in canola and Indian mustard in the lower rainfall areas of Western Australia. *Australian Journal of Agricultural Research* **55**: 367–377.

Sinaki JM, Heravan EM, Rad AHS, Noormohammadi G, Zarei G. 2007. The effects of water deficit during growth stages of canola (*Brassica napus* L.). *Journal of Agriculture and Environmental Sciences* **2**: 417–422.

Sirichandra C, Davanture M, Turk BE, Zivy M, Valot B, Leung J, Merlot S. 2010. The Arabidopsis ABA-activated kinase OST1 phosphorylates the bZIP transcription factor ABF3 and creates a 14-3-3 binding site involved in its turnover. *PLoS ONE* **5**: e13935.

Sirichandra C, Gu D, Hu HC, Davanture M, Lee S, Djaoui M, Valotc B, Zivyc M, Leunga J, Merlot S *et al.* 2009. Phosphorylation of the Arabidopsis AtrbohF NADPH oxidase by OST1 protein kinase. *FEBS Letters* **583**: 2982–2986.

Staxén I, Pical C, Montgomery LT, Gray JE, Hetherington AM, McAinsh MR. 1999. Abscisic acid induces oscillations in guard-cell cytosolic free calcium that involve phosphoinositide-specific phospholipase C. *Proceedings of the National Academy of Sciences, USA* **96**: 1779–1784.

Sunkar R, Li YF, Jagadeeswaran G. 2012. Functions of microRNAs in plant stress responses. *Trends in Plant Science* **17**: 196–203.

The *Brassica rapa* Genome Sequencing Project Consortium. 2011. The genome of the mesopolyploid crop species *Brassica rapa*. *Nature Genetics* **43**: 1035–1039.

Turner NC. 2001. Optimizing water use. In: Nosberger J, Geiger HH, Struik PC, eds. *Crop science: progress and prospects.* Wallingford, UK: CABI International, 119–135.

Ullah F, Bano A, Nosheen A. 2012. Effects of plant growth regulators on growth and oil quality of canola (*Brassica napus L.*) under drought stress. *Pakistan Journal of Botany* **44**: 1873–1880.

Umezawa T, Nakashima K, Miyakawa T, Kuromori T, Tanokura M, Shinozaki K, Yamaguchi-Shinozaki K. 2010. Molecular basis of the core regulatory network in ABA responses: sensing, signaling and transport. *Plant and Cell Physiology* **51**: 1821–1839.

Umezawa T, Sugiyama N, Takahashi F, Anderson JC, Ishihama Y, Peck SC, Shinozaki K. 2013. Genetics and phosphoproteomics reveal a protein phosphorylation network in the abscisic acid signaling pathway in *Arabidopsis thaliana*. *Science Signaling* **6**: rs8.

Vartanian N, Damerval C, Vienne D. 1987. Drought-induced changes in protein patterns of *Brassica napus* var. oleifera roots. *Plant Physiology* **84**: 989–992.

Verkest A, Byzova M, Martens C, Willems P, Verwulgen T, Slabbinck B, Rombaut D, Van de Velde J, Vandepoele K, Standaert E *et al.* 2015. Selection for improved energy use efficiency and drought tolerance in canola results in distinct transcriptome and epigenome changes. *Plant Physiology* **168**: 1338–1350.

Wan J, Griffiths R, Ying J, McCourt P, Huang Y. 2009. Development of drought-tolerant canola (*Brassica napus* L.) through genetic modulation of ABA-mediated stomatal responses. *Crop Science* **49**: 1539–1554.

Wang J, Lydiate D, Parkin I, Falentin C, Delourme R, Carion P, King G. 2011a. Integration of linkage maps for the amphidiploid *Brassica napus* and comparative mapping with *Arabidopsis* and *Brassica rapa*. *BMC Genomics* **12**: 101.

Wang JM, Fan Z, Liu Z, Xiang J, Chai L, Li X, Yang Y. 2011b. Thylakoid-bound ascorbate peroxidase increases resistance to salt stress and drought in *Brassica napus*. *African Journal of Biotechnology* **10**: 8039–8045.

Wang M, Yuan F, Hao H, Zhang Y, Zhao H, Guo A, Hu J, Zhou X, Xie C. 2013. *BolOST1*, an ortholog of *Open Stomata 1* with alternative splicing products in *Brassica oleracea*, positively modulates drought responses in plants. *Biochemical and Biophysical Research Communications* **442**: 214–220.

Wang RS, Pandey S, Li S, Gookin TE, Zhao Z, Albert R, Assmann SM. 2011c. Common and unique elements of the ABA-regulated transcriptome of *Arabidopsis* guard cells. *BMC Genomics* **12**: 216.

Wang T, Chen L, Zhao M, Tian Q, Zhang WH. 2011d. Identification of drought-responsive microRNAs in *Medicago truncatula* by genome-wide high-throughput sequencing. *BMC Genomics* **12**: 367.

Wang XQ, Ullah H, Jones AM, Assmann SM. 2001. G protein regulation of ion channels and abscisic acid signaling in *Arabidopsis* guard cells. *Science* **292**: 2070–2072.

Wang Y, Beaith M, Chalifoux M, Ying J, Uchacz T, Sarvas C, Griffiths R, Kuzma M, Wan J, Huang Y. 2009. Shoot-specific down-regulation of protein farnesyltransferase (alpha-subunit) for yield protection against drought in canola. *Molecular Plant* **2**: 191–200.

Wang Y, Ying J, Kuzma M, Chalifoux M, Sample A, McArthur C, Uchacz T, Sarvas C, Wan J, Dennis DT *et al.* 2005. Molecular tailoring of farnesylation for plant drought tolerance and yield protection. *Plant Journal* **43**: 413–424.

White JW, Castillo JA. 1989. Relative effect of root and shoot genotypes on yield of common bean under drought stress. *Crop Science* **29**: 360–362.

Willenborg C, Gulden R, Johnson E, Shirtliffe S. 2004. Canola: germination characteristics of polymer-coalted canola (*Brassica napus* L.) seeds subjected to moisture stress at different temperatures. *Agronomy Journal* **96**: 786–791.

Wolfe MD, Tonsor SJ. 2014. Adaptation to spring heat and drought in northeastern Spanish *Arabidopsis thaliana*. *New Phytologist* **201**: 323–334.

Wu J, Li F, Xu K, Gao G, Chen B, Yan G. 2014. Assessing and broadening genetic diversity of a rapeseed germplasm collection. *Breeding Science* **64**: 321.

Xie F, Huang S, Guo K, Xiang A, Zhu Y, Nie L, Yang Z. 2007. Computational identification of novel microRNAs and targets in *Brassica napus*. *FEBS Letters* **581**: 1464–1474.

Yang C, Zhang X, Zou C, Cheng Y, Zheng P, Li G. 2007. Effects of drought simulated by PEG-6000 on germination and seedling growth of rapeseed (*Brassica napus* L.). *Chinese Journal of Oil Crop Science* **29**: 425–430.

Yang J, Osman K, Iqbal M, Stekel DJ, Luo Z, Armstrong SJ, Franklin FCH. 2012. Inferring the *Brassica rapa* interactome using protein-protein interaction data from *Arabidopsis thaliana*. *Frontiers in Plant Science* **3**: 297.

Yang M, Yang Q, Fu T, Zhou Y. 2011. Overexpression of the *Brassica napus BnLAS* gene in *Arabidopsis* affects plant development and increases drought tolerance. *Plant Cell Reports* **30**: 373–388.

Yang Y, Costa A, Leonhardt N, Siegel RS, Schroeder JI. 2008. Isolation of a strong *Arabidopsis* guard cell promoter and its potential as a research tool. *Plant Methods* **4**: 6.

Ying L, Chen H, Cai W. 2014. *BnNAC485* is involved in abiotic stress responses and flowering time in *Brassica napus*. *Plant Physiology and Biochemistry* **79**: 77–87.

Yong HY, Wang C, Bancroft I, Li F, Wu X, Kitashiba H, Nishio T. 2015. Identification of a gene controlling variation in the salt tolerance of rapeseed (*Brassica napus* L.). *Planta* **242**: 313–326.

Yu Q, Hu Y, Li J, Wu Q, Lin Z. 2005. Sense and antisense expression of plasma membrane aquaporin *BnPIP1* from *Brassica napus* in tobacco and its effects on plant drought resistance. *Plant Science* **169**: 647–656.

Yu S, Zhang F, Yu Y, Zhang D, Zhao X, Wang W. 2012. Transcriptome profiling of dehydration stress in the Chinese cabbage (*Brassica rapa* L. ssp pekinensis) by tag sequencing. *Plant Molecular Biology Reporter* **30**: 17–28.

Yuan F, Wang M, Hao H, Zhang Y, Zhao H, Guo A, Xu H, Zhou X, Xie C. 2013. Negative regulation of abscisic acid signaling by the *Brassica oleracea ABI1* ortholog. *Biochemical and Biophysical Research Communications* **442**: 202–208.

Zhang J, Creelman RA, Zhu J. 2004. From laboratory to field. Using information from *Arabidopsis* to engineer salt, cold, and drought tolerance in crops. *Plant Physiology* **135**: 615–621.

Zhang Y, Zhu H, Zhang Q, Li M, Yan M, Wang R, Wang L, Welti R, Zhang W, Wang X. 2009. Phospholipase Dα1 and phosphatidic acid regulate NADPH oxidase activity and production of reactive oxygen species in ABA-mediated stomatal closure in *Arabidopsis*. *Plant Cell* **21**: 2357–2377.

Zhao Y, Wang M, Fu S, Yang W, Qi C, Wang X. 2012. Small RNA profiling in two *Brassica napus* cultivars identifies microRNAs with oil production-and development-correlated expression and new small RNA classes. *Plant Physiology* **158**: 813–823.

Zhou X, Yuan F, Wang M, Guo A, Zhang Y, Xie C. 2013. Molecular characterization of an *ABA insensitive 5* orthologue in *Brassica oleracea*. *Biochemical and Biophysical Research Communications* **430**: 1140–1146.

Zhou Z, Song J, Yang Z. 2012. Genome-wide identification of *Brassica napus* microRNAs and their targets in response to cadmium. *Journal of Experimental Botany* **63**: 4597–4613.

Zhu M, Dai S, McClung S, Yan X, Chen S. 2009. Functional differentiation of *Brassica napus* guard cells and mesophyll cells revealed by comparative proteomics. *Molecular & Cellular Proteomics* **8**: 752–766.

Zhu M, Simons B, Zhu N, Oppenheimer DG, Chen S. 2010. Analysis of abscisic acid responsive proteins in *Brassica napus* guard cells by multiplexed isobaric tagging. *Journal of Proteomics* **73**: 790–805.

Zhu M, Zhu N, Song W, Harmon AC, Assmann SM, Chen S. 2014. Thiol-based redox proteins in *Brassica napus* guard cell abscisic acid and methyl jasmonate signaling. *Plant Journal* **78**: 491–515.

Acknowledgements

Appendix A, in full, is a reprint of the material as it appears in New Phytologist, 2016. Zhu M, Monroe JG, Suhail Y, Villiers F, Mullen J, Pater D, Hauser F, Jeon BW, Bader JS, Kwak JM, Schroeder, JI, McKay, JK, and Assmann SM. The dissertation author was a co-author of this paper.

Appendix B

Targeted knockdown of Clade A protein phosphatases (PP2Cs)

for increased drought tolerance in canola (*Brassica napus*)

Introduction

When plants perceive drought stress, they respond by a variety of methods, including escape, avoidance, and tolerance. One of the best understood avoidance and tolerance responses is the abscisic acid (ABA) signal transduction pathway. Abiotic stresses, including drought, elicit production of the plant hormone (ABA), which stimulates stomatal closure and alters gene expression, increasing the plant's tolerance to the stress.

Upon sensing drought stress, endogenous ABA levels increase, initiating a complex signal transduction pathway which allows the plant to modulate stomatal aperture, reducing transpirational water loss. (Cutler *et al.*, 2010; Hubbard *et al.*, 2010; Munemasa *et al.*, 2015). ABA binds to the "PYR/PYL/RCAR" receptors, inducing a conformational change which allows interaction with the type 2C protein phosphatases (PP2Cs) of the clade A subfamily (Park *et al.*, 2009; Klingler, Batelli and Zhu, 2010; Joshi-Saha, Valon and Leung, 2011). Clade A plant PP2Cs act as negative regulators of ABA signaling, with nine identified Clade A PP2Cs implicated in the ABA signaling pathway. PP2Cs characterized in Clade A include ABI1, ABI2, HAB1, HAB2, and PP2CA (Schweighofer, Hirt and Meskiene, 2004; Bhaskara, Nguyen and Verslues, 2012).

PP2Cs act by both physical interaction with and dephosphorylation of downstream kinases of the SnRK2 family (Hubbard *et al.*, 2010). After ABA has bound to the PYR/PYL/RCAR receptors, a Trp residue on the PP2C interacts with the receptor-ABA complex, locking the molecules together and preventing phosphatase activity (Melcher *et al.*, 2009; Miyazono *et al.*, 2009; Yin *et al.*, 2009). This allows phosphorylation of the

SnRK2 and CDPK kinases (Fujii *et al.*, 2009; Fujii and Zhu, 2012), which then phosphorylate downstream targets such as transcription factors and ion channels (e.g. SLAC1) which are involved in stomatal closure (Cutler *et al.*, 2010; Brandt *et al.*, 2012, 2015; Umezawa *et al.*, 2013; Munemasa *et al.*, 2015).

Previous studies have shown that while single gene knockouts of PP2Cs have limited effect on ABA responses, inactivation of multiple PP2Cs involved in ABA signaling improves drought tolerance (Saez *et al.*, 2006; Rubio *et al.*, 2009). Saez et al., showed mutants with double PP2C knockouts (*hab1-1 abi1-2,* and *hab1-1 abi1-3*) had an ABA-hypersensitive phenotype in growth assays and stomatal closure. The mutants also showed reduced water consumption, but demonstrated decreased biomass and yield under non-drought conditions. Similar results were found for PP2C triple loss of function mutants (*hab1-1 abi1-2 abi2-2* and *hab1-1 abi1-2 pp2ca-1)* (Rubio *et al.*, 2009).

This research seeks to determine the effect of targeted PP2C knockdown on plant responses to drought stress. Despite the high sequence similarity between *Arabidopsis* and *Brassica* transcripts and corresponding proteins, these genes are generally triplicated in *Brassica* as compared to *Arabidopsis* (J. Wang *et al.*, 2011; Chalhoub *et al.*, 2014; Cheng *et al.*, 2016). RNAi technology can be used to target multiple genes within a gene family without requiring individual gene mutations (Kerschen *et al.*, 2004; Bezanilla *et al.*, 2005). As whole-plant knockouts result in constitutive growth inhibition under non-drought conditions, it was hypothesized that by inhibiting PP2Cs specifically in guard cells or in a manner induced by drought, drought responses can be enhanced without the corresponding growth penalty.

Results

B. napus homologues were identified for target PP2Cs (ABI1, ABI2, HAB1, PP2CA) via the Brassica Database (Cheng *et al.*, 2011). The target region chosen was a 400 bp region in the highly conserved PP2C catalytic site (Bork *et al.*, 1996; Rodriguez, 1998). Pairwise alignment analysis was performed using the ClustalW program to find highly homologous regions between the *B. napus* genes and also the *A. thaliana* cDNAs to identify RNAi target regions for silencing of these negative regulators of drought-induced ABA signaling (Figure B1).

The RNAi target sequence was compared for sequence similarity to the published *B. napus* genome using the Brassica Database (BRAD, http://brassicadb.org/brad/index.php). The target sequence had the highest sequence similarity to BnABI1 (chrA01 88%, chrC01 87.8% identity) and BnABI2 (chrA10 77.6% identity). Lower sequence similarity was found to the A chromosome copy of HAB1 (chrA07 51.6% identity) and both PP2CA copies (chrA05 44%, chrC05 43.6% identity). The lowest similarity was calculated for the C chromosome copy of HAB1 (26.5% identity). HAB2, which is found in *A. thaliana* and other *Brassica* species (Kerk, 2002; Schweighofer, Hirt and Meskiene, 2004; Cheng *et al.*, 2011; X. Wang *et al.*, 2011; Ludwikõw *et al.*, 2013), has not been identified in *B. napus* (Chalhoub *et al.*, 2014; Babula-Skowrońska *et al.*, 2015).

Hairpin RNAi constructs of identified PP2Cs (ABI1, ABI2, HAB1, PP2CA) were designed under the tissue-specific promoter *pGC1* (At1g22690), which drives strong and preferential gene expression in guard cells (Yang *et al.*, 2008), including in *Brassica*

(Figure B2). The RNAi construct was also driven under the stress-inducible promoter *rd29a* (Ishitani *et al.*, 1997) to investigate and compare responses of whole plant down-regulation of the PP2Cs under drought stress, while minimizing negative growth effects of ABA under non-drought conditions (Saez *et al.*, 2006; Rubio *et al.*, 2009). The construct was also driven under the whole-plant expressed 35S promoter to compare to whole plant knockdown of PP2Cs.

The RNAi constructs were transformed into *Brassica napus* using callus transformation. We obtained a total of 12 independent *Brassica napus* lines containing the *rd29a*-driven anti-PP2C constructs. We also obtained 6 independent *Arabidopsis thaliana* lines containing the *rd29a*-driven PP2C RNAi construct. The independent *A. thaliana* transformants all showed stunted growth and a developmental phenotype of multiple rosettes (Figure B3). We were unable to obtain any lines containing the 35S-driven construct or the *pGC1*-driven construct in either *B. napus* or in *A. thaliana*.

This method of introducing RNAi for *Brassica* PP2Cs had unreliable results. We were not able to obtain positive constructs driven by the 35S promoter. It is possible the RNAi knockdown is lethal when expressed on a whole-plant level. As the region chosen for knockdown was within the well-conserved catalytic domain of PP2Cs, the whole-plant knockdown may have had off-target effects upon other PP2Cs uninvolved in the ABA signaling pathway.

Materials and Methods

Construction of plant expression vector

For the PP2C RNAi construct, the coding sequences of known *A. thaliana* and *B. napus* PP2Cs were aligned ClustalW alignment using Geneious R6 software (Kearse *et al.*, 2012). The region of highest sequence consensus was analyzed to correspond to the catalytic domain of the proteins. A 400-bp consensus sequence was cloned downstream of the 35S promoter of the *Brassica* optimized plasmid, pBRACT507 (John Innes, Norwich, UK) using Gateway cloning technology (Invitrogen, Carlsbad, CA) (Smedley and Harwood, 2014). For subsequent vectors, the promoter region of the plasmid containing the RNAi target sequence was replaced by the *pGC1* or *rd29a* promoters, respectively. The expression vectors were introduced into *Agrobacterium tumefaciens* GV3101 containing pSOUP helper plasmid through electroporation.

RNAi target sequence

TTGTACGGCGTGACTTCCATCTGTGGAAGAAGACCGGAGATGGAAGATGCTCTCTCCG
CGATACCAAGATTCCTCCAATCTCCGACCAATTCGTTGATAGATGGTCGTTTCAATCCTCAGTCC
GCCGCTCACTTCTTCGGCGTCTACGACGGCCACGGCGGTTCTCAGGTAGCGAACTATTGCAGAG
AGAGGATGCACTTGGCTTTAGCGGAGGAGATAGAGAAGGAGAAACCGATGCTC

Plant transformation

Spring-type oilseed rape (*B. napus* cv. Westar) transformants were generated by the method described by (Yao *et al.*, 2016) at Huazhong Agricultural University (Wuhan,

China). Plasmids were transformed into *A. thaliana* (Col-0 ecotype) by the floral dip method (Clough and Bent, 1998). Seeds were grown on 1/2 -MS phytoagar plates containing Kanamycin (50 µg/mL) to choose positive transformants.

Analysis of transgenic plants by PCR

Plant genomic DNA was extracted from collected samples of both *Brassica* and *Arabidopsis* using the method of (Edwards, Johnstone and Thompson, 1991). Non-transformed *Brassica* and *Arabidopsis* DNA were used as a negative control. Putative transformants were detected by PCR screening using primers targeting plasmid backbone and promoter region.

PCR genotyping primers

35S Forward: TAATACGACTCACTATAGGG

35S Reverse: TAGCTGGGCAATGGAATCCG

pGC1 Forward: ATGGTTGCAACAGAGAGGA

pGC1 Reverse: ATTTCTTGAGTAGTGATTTTGAAGTAG

rd29a Forward: TAATACGACTCACTATAGGG

rd29a Reverse: TTGCTCTCTACGCGTGTCTG

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REFERENCES

Babula-Skowrońska, D., Ludwików, A., Cieśla, A., Olejnik, A., Cegielska-Taras, T., Bartkowiak-Broda, I. and Sadowski, J. (2015) 'Involvement of genes encoding ABI1 protein phosphatases in the response of Brassica napus L. to drought stress', Plant Molecular Biology, 88(4–5), pp. 445–457. doi: 10.1007/s11103-015-0334-x.

Bezanilla, M., Perroud, P.-F., Pan, A., Klueh, P. and Quatrano, R. S. (2005) 'An RNAi system in Physcomitrella patens with an internal marker for silencing allows for rapid identification of loss of function phenotypes.', Plant biology (Stuttgart, Germany), 7(3), pp. 251–7. doi: 10.1055/s-2005-837597.

Bhaskara, G. B., Nguyen, T. T. and Verslues, P. E. (2012) 'Unique drought resistance functions of the highly ABA-induced clade A protein phosphatase 2Cs.', Plant physiology, 160(1), pp. 379–95. doi: 10.1104/pp.112.202408.

Bork, P., Brown, N. P., Hegyi, H. and Schultz, J. (1996) 'The protein phosphatase 2C (PP2C) superfamily: Detection of bacterial homologues', Protein Science, 5, pp. 1421–1425.

Brandt, B., Brodsky, D. E., Xue, S., Negi, J., Iba, K., Kangasjärvi, J., Ghassemian, M., Stephan, A. B., Hu, H. and Schroeder, J. I. (2012) 'Reconstitution of abscisic acid activation of SLAC1 anion channel by CPK6 and OST1 kinases and branched ABI1 PP2C phosphatase action.', Proceedings of the National Academy of Sciences of the United States of America, 109(26), pp. 10593–8. doi: 10.1073/pnas.1116590109.

Brandt, B., Munemasa, S., Wang, C., Nguyen, D., Yong, T., Yang, P. G., Poretsky, E., Belknap, T. F., Waadt, R., Alemań, F. and Schroeder, J. I. (2015) 'Calcium specificity signaling mechanisms in abscisic acid signal transduction in Arabidopsis guard cells', eLife, 4(JULY 2015), pp. 1–25. doi: 10.7554/eLife.03599.

Chalhoub, B., Denoeud, F., Liu, S., Parkin, I. A. P., Tang, H., Wang, X., Chiquet, J., Belcram, H., Tong, C., Samans, B., Correa, M., Da Silva, C., Just, J., Falentin, C., Koh, C. S., Le Clainche, I., Bernard, M., Bento, P., Noel, B., Labadie, K., Alberti, A., Charles, M., Arnaud, D., Guo, H., Daviaud, C., Alamery, S., Jabbari, K., Zhao, M., Edger, P. P., Chelaifa, H., Tack, D., Lassalle, G., Mestiri, I., Schnel, N., Le Paslier,

M.-C., Fan, G., Renault, V., Bayer, P. E., Golicz, A. A., Manoli, S., Lee, T.-H., Thi, V. H. D., Chalabi, S., Hu, Q., Fan, C., Tollenaere, R., Lu, Y., Battail, C., Shen, J., Sidebottom, C. H. D., Wang, X., Canaguier, A., Chauveau, A., Berard, A., Deniot, G., Guan, M., Liu, Z., Sun, F., Lim, Y. P., Lyons, E., Town, C. D., Bancroft, I., Wang, X., Meng, J., Ma, J., Pires, J. C., King, G. J., Brunel, D., Delourme, R., Renard, M., Aury, J.-M., Adams, K. L., Batley, J., Snowdon, R. J., Tost, J., Edwards, D., Zhou, Y., Hua, W., Sharpe, A. G., Paterson, A. H., Guan, C. and Wincker, P. (2014) 'Early allopolyploid evolution in the post-Neolithic Brassica napus oilseed genome', Science, 345(6199), pp. 950–953. doi: 10.1126/science.1253435.

Cheng, F., Liu, S., Wu, J., Fang, L., Sun, S., Liu, B., Li, P., Hua, W. and Wang, X. (2011) 'BRAD, the genetics and genomics database for Brassica plants.', BMC Plant Biology. BioMed Central Ltd, 11(1), pp. 136–142. doi: 10.1186/1471-2229-11-136.

Cheng, F., Sun, R., Hou, X., Zheng, H., Zhang, F., Zhang, Y., Liu, B., Liang, J., Zhuang, M., Liu, Y., Liu, D., Wang, X., Li, P., Liu, Y., Lin, K., Bucher, J., Zhang, N., Wang, Y., Wang, H., Deng, J., Liao, Y., Wei, K., Zhang, X., Fu, L., Hu, Y., Liu, J., Cai, C., Zhang, S., Zhang, S., Li, F., Zhang, H., Zhang, J., Guo, N., Liu, Z., Liu, J., Sun, C., Ma, Y., Zhang, H., Cui, Y., Freeling, M. R., Borm, T., Bonnema, G., Wu, J. and Wang, X. (2016) 'Subgenome parallel selection is associated with morphotype diversification and convergent crop domestication in Brassica rapa and Brassica oleracea', Nature Genetics, 48(10), pp. 1218–1224. doi: 10.1038/ng.3634.

Clough, S. J. and Bent, A. F. (1998) 'Floral dip: A simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana', Plant Journal, 16(6), pp. 735–743. doi: 10.1046/j.1365-313X.1998.00343.x.

Cutler, S. R., Rodriguez, P. L., Finkelstein, R. R. and Abrams, S. R. (2010) 'Abscisic acid: emergence of a core signaling network', Annual Reviews of Plant Biology, 61, pp. 651–679.

Edwards, K., Johnstone, C. and Thompson, C. (1991) 'A simple and rapid method for the preparation of plant genomic DNA for PCR analysis.', Nucleic acids research, 19(6), p. 1349.

Fujii, H., Chinnusamy, V., Rodrigues, A., Rubio, S., Antoni, R., Park, S.-Y., Cutler, S. R., Sheen, J., Rodriguez, P. L. and Zhu, J.-K. (2009) 'In vitro reconstitution of an abscisic acid signalling pathway.', Nature. Nature Publishing Group, 462(7273), pp. 660–4. doi: 10.1038/nature08599.

Fujii, H. and Zhu, J.-K. (2012) 'Osmotic stress signaling via protein kinases.', Cellular and molecular life sciences: CMLS, 69(19), pp. 3165–73. doi: 10.1007/s00018-012-1087-1.

Hubbard, K. E., Nishimura, N., Hitomi, K., Getzoff, E. D. and Schroeder, J. I. (2010) 'Early abscisic acid signal transduction mechanisms: newly discovered components and newly emerging questions.', Genes & development, 24(16), pp. 1695–708. doi: 10.1101/gad.1953910.

Ishitani, M., Xiong, L., Stevenson, B. and Zhu, J. K. (1997) 'Genetic analysis of osmotic and cold stress signal transduction in Arabidopsis: interactions and convergence of abscisic acid-dependent and abscisic acid-independent pathways.', The Plant cell, 9(11), pp. 1935–49. doi: 10.1105/tpc.9.11.1935.

Joshi-Saha, A., Valon, C. and Leung, J. (2011) 'A brand new START: abscisic acid perception and transduction in the guard cell.', Science signaling, 4(201), p. re4. doi: 10.1126/scisignal.2002164.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Meintjes, P. and Drummond, A. (2012) 'Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data', Bioinformatics, 28(12), pp. 1647–1649. doi: 10.1093/bioinformatics/bts199.

Kerk, D. (2002) 'The Complement of Protein Phosphatase Catalytic Subunits Encoded in the Genome of Arabidopsis', Plant Physiology, 129(2), pp. 908–925. doi: 10.1104/pp.004002.

Kerschen, A., Napoli, C. A., Jorgensen, R. A. and Müller, A. E. (2004) 'Effectiveness of RNA interference in transgenic plants', FEBS Letters, 566(1–3), pp. 223–228. doi: 10.1016/j.febslet.2004.04.043.

Klingler, J. P., Batelli, G. and Zhu, J.-K. (2010) 'ABA receptors: the START of a new paradigm in phytohormone signalling.', Journal of experimental botany, 61(12), pp. 3199–210. doi: 10.1093/jxb/erq151.

Ludwikõw, A., Babula-Skowrońska, D., Szczepaniak, M., Belter, N., Dominiak, E. and Sadowski, J. (2013) 'Expression profiles and genomic organisation of group A protein phosphatase 2C genes in Brassica oleracea', Annals of Applied Biology, 163, pp. 124–134. doi: 10.1111/aab.12039.

Melcher, K., Ng, L.-M., Zhou, X. E., Soon, F.-F., Xu, Y., Suino-Powell, K. M., Park, S.-Y., Weiner, J. J., Fujii, H., Chinnusamy, V., Kovach, A., Li, J., Wang, Y., Li, J., Peterson, F. C., Jensen, D. R., Yong, E.-L., Volkman, B. F., Cutler, S. R., Zhu, J.-K. and Xu, H. E. (2009) 'A gate-latch-lock mechanism for hormone signalling by abscisic acid receptors.', Nature. Nature Publishing Group, 462(7273), pp. 602–8. doi: 10.1038/nature08613.

Miyazono, K., Miyakawa, T., Sawano, Y., Kubota, K., Kang, H.-J., Asano, A., Miyauchi, Y., Takahashi, M., Zhi, Y., Fujita, Y., Yoshida, T., Kodaira, K.-S., Yamaguchi-Shinozaki, K. and Tanokura, M. (2009) 'Structural basis of abscisic acid signalling', Nature. Nature Publishing Group, 462(7273), pp. 609–614. doi: 10.1038/nature08583.

Munemasa, S., Hauser, F., Park, J., Waadt, R., Brandt, B. and Schroeder, J. I. (2015) 'Mechanisms of abscisic acid-mediated control of stomatal aperture', Current Opinion in Plant Biology. doi: 10.1016/j.pbi.2015.10.010.

Park, S., Fung, P., Nishimura, N., Jensen, D. R., Fujii, H., Zhao, Y., Lumba, S., Santiago, J., Rodrigues, A., Chow, T. F., Alfred, S. E., Bonetta, D., Finkelstein, R., Provart, N. J., Desveaux, D., Rodriguez, P. L., Mccourt, P., Zhu, J., Schroeder, J. I., Volkman, B. F. and Cutler, S. R. (2009) 'Abscisic Acid Inhibits Type 2C Protein Phosphatases via the PYR/PYL Family of START Proteins', Science, 324(April), pp. 1068–1071.

Rodriguez, P. L. (1998) 'Protein phosphatase 2C (PP2C) function in higher plants', Plant Molecular Biology, 38(6), pp. 919–927. doi: 10.1023/A:1006054607850.

Rubio, S., Rodrigues, A., Saez, A., Dizon, M. B., Galle, A., Kim, T.-H., Santiago, J., Flexas, J., Schroeder, J. I. and Rodriguez, P. L. (2009) 'Triple loss of function of protein phosphatases type 2C leads to partial constitutive response to endogenous abscisic acid.', Plant physiology, 150(3), pp. 1345–55. doi: 10.1104/pp.109.137174.

Saez, A., Robert, N., Maktabi, M. H., Schroeder, J. I., Serrano, R. and Rodriguez, P. L. (2006) 'Enhancement of abscisic acid sensitivity and reduction of water consumption in Arabidopsis by combined inactivation of the Protein Phosphatases Type 2C ABI1', Plant Physiology, 141(August), pp. 1389–1399. doi: 10.1104/pp.106.081018.ulation.

Schweighofer, A., Hirt, H. and Meskiene, I. (2004) 'Plant PP2C phosphatases: emerging functions in stress signaling.', Trends in plant science, 9(5), pp. 236–43. doi: 10.1016/j.tplants.2004.03.007.

Smedley, M. A. and Harwood, W. A. (2014) 'Gateway-compatible plant transformation vectors', in Agrobacterium Protocols: Third Edition, pp. 1–368. doi: 10.1007/978-1-4939-1695-5.

Umezawa, T., Sugiyama, N., Takahashi, F., Anderson, J. C., Ishihama, Y., Peck, S. C. and Shinozaki, K. (2013) 'Genetics and phosphoproteomics reveal a protein phosphorylation network in the abscisic acid signaling pathway in Arabidopsis thaliana', Science Signaling, 6(270), p. rs8-rs8. doi: 10.1126/scisignal.2003509.

Wang, J., Lydiate, D. J., Parkin, I. a P., Falentin, C., Delourme, R., Carion, P. W. C. and King, G. J. (2011) 'Integration of linkage maps for the Amphidiploid Brassica napus and comparative mapping with Arabidopsis and Brassica rapa.', BMC genomics. BioMed Central Ltd, 12(1), p. 101. doi: 10.1186/1471-2164-12-101.

Wang, X., Wang, H., Wang, J., Sun, R., Wu, J., Liu, S., Bai, Y., Mun, J.-H., Bancroft, I., Cheng, F., Huang, S., Li, X., Hua, W., Wang, J., Wang, X., Freeling, M., Pires, J. C., Paterson, A. H., Chalhoub, B., Wang, B., Hayward, A., Sharpe, A. G., Park, B.-S., Weisshaar, B., Liu, B., Li, B., Liu, B., Tong, C., Song, C., Duran, C., Peng, C., Geng, C., Koh, C., Lin, C., Edwards, D., Mu, D., Shen, D., Soumpourou, E., Li, F., Fraser, F., Conant, G., Lassalle, G., King, G. J., Bonnema, G., Tang, H., Wang, H., Belcram, H., Zhou, H., Hirakawa, H., Abe, H., Guo, H., Wang, H., Jin, H., Parkin, I. a P., Batley, J., Kim, J.-S., Just, J., Li, J., Xu, J., Deng, J., Kim, J. a, Li, J., Yu, J., Meng, J., Wang, J., Min, J., Poulain, J., Hatakeyama, K., Wu, K., Wang, L., Fang, L., Trick, M., Links, M. G., Zhao, M., Jin, M., Ramchiary, N., Drou, N., Berkman, P. J., Cai, Q., Huang, Q., Li, R., Tabata, S., Cheng, S., Zhang, S., Zhang, S., Huang, S., Sato, S., Sun, S., Kwon, S.-J., Choi, S.-R., Lee, T.-H., Fan, W., Zhao, X., Tan, X., Xu, X., Wang, Y., Qiu, Y., Yin, Y., Li, Y., Du, Y., Liao, Y., Lim, Y., Narusaka, Y., Wang, Y., Wang, Z., Li, Z., Wang, Z., Xiong, Z. and Zhang, Z. (2011) 'The genome of the mesopolyploid crop species Brassica rapa.', Nature genetics, 43(10), pp. 1035–9. doi: 10.1038/ng.919.

Yang, Y., Costa, A., Leonhardt, N., Siegel, R. S. and Schroeder, J. I. (2008) 'Isolation of a strong Arabidopsis guard cell promoter and its potential as a research tool.', Plant methods, 4, p. 6. doi: 10.1186/1746-4811-4-6.

Yao, X., Wang, Y., Yue, X., Liu, M. and Liu, K. (2016) 'Generation of tribenuron-methyl herbicide-resistant OsCYP81A6-expressing rapeseed (Brassica napus L.) plants for hybrid seed production using chemical-induced male sterility', Plant Breeding, 135(3), pp. 349–354. doi: 10.1111/pbr.12361.

Yin, P., Fan, H., Hao, Q., Yuan, X., Wu, D., Pang, Y., Yan, C., Li, W., Wang, J. and Yan, N. (2009) 'Structural insights into the mechanism of abscisic acid signaling by PYL proteins.', Nature structural & molecular biology, 16(12), pp. 1230–6. doi: 10.1038/nsmb.1730.

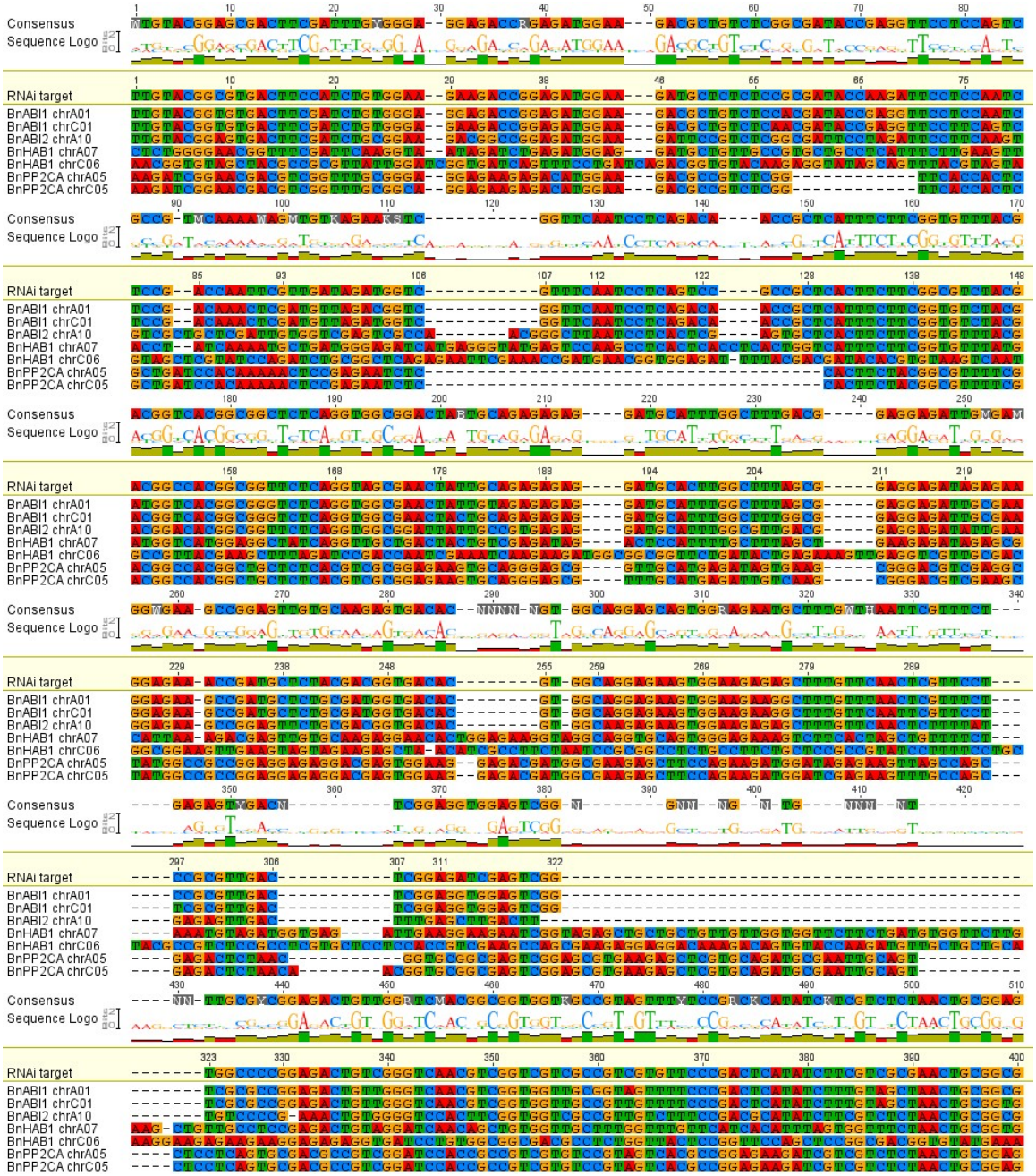

Figure B1. RNAi target consensus Comparison of RNAi target sequence (first line) to six published *B. napus* PP2C genes ABI1, ABI2, HAB1, and PP2CA. RNAi target sequence had highest consensus with ABI1 and ABI2.

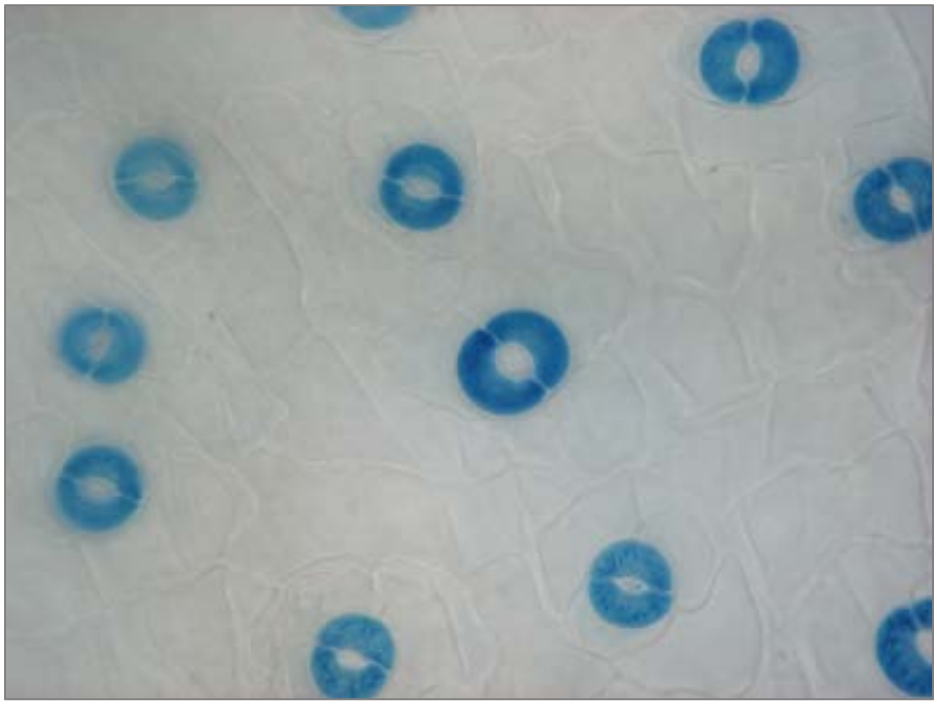

Figure B2. Guard cell specific staining using a pGC1::GUS construct in Brassica (Schroeder lab, unpublished data).

Figure B3. *A. thaliana* RNAi transformants. Examples of T1 generation *Arabidopsis* displaying multi-rosette phenotype. Arrows indicate aerial rosettes. Similar findings were observed in 6 independent transformants.

Appendix C

Rapid drydown protocol for leaf temperature phenotyping of drought responses

Introduction

Drought is one of the most damaging of all abiotic stresses, in terms of loss to crop productivity. Breeding efforts to improve drought tolerance are limited and slowed both by the complexity of plant responses to drought and by the variation in drought intensity, timing, and length. Drought tolerance refers to several traits, for which numerous genes and interactions may be involved. This complexity is increased by gene interactions which are subject to transcriptional and post-transcriptional regulation (Belostotsky and Rose, 2005) and by metabolic fluxes (Morandini and Salamini, 2003). To overcome reductions in crop yields from drought, plant breeders must understand not only what genes and traits are involved in plant responses, but also how identified traits are expressed under different growth conditions.

Breeding for improved water use efficiency needs to address specific requirements at the main stages of the plant life cycle, including: germination and seedling establishment, vegetative development, flowering, and grain filling. To do this, researchers need to be able to identify phenotypes under not only severe water stress, but also over periods of varied intensity and length. Researchers are challenged to develop effective and inexpensive ways to impose controlled conditions to identify promising phenotypes at specific developmental stages and/or level of drought stress. High-throughput infrared imaging can be an effective tool to analyze physiological changes in response to drought over time (Merlot *et al.*, 2002; Verslues *et al.*, 2006; Berger, Parent and Tester, 2010)

To address this question, we developed a water limitation and drought phenotyping protocol which allows for rapid water loss of approximately 10% soil water content loss per day. The non-destructive imaging of shoots allows for monitoring of the same plants throughout the experiment and collection of biomass following the treatment. This technique also allows for temporal resolution related to soil moisture content.

Results

For this experiment, *Arabidopsis thaliana* lines overexpressing carbonic anhydrases (CA) identified to regulate CO_2 controlled stomatal movements (Hu *et al.*, 2010) were tested over an extended drought and recovery regimen. Col-0, carbonic anhydrase double knockout mutants (*ca1ca4*) and *ost1-1* mutants were included as controls. Plants were grown in Profile clay soil ("Profile Porous Ceramic (PPC) "Greens Grade" soil, Profile Products LLC, Buffalo Grove, IL) for rapid soil drydown and soil moisture measured directly using a soil moisture probe to obtain accurate soil moisture measurements. Thermal and soil moisture measurements began 6 weeks post-germination. The soil had a uniform soil moisture change under drydown conditions for all tested lines (Figure C1).

Thermal imaging data were used to determine an average temperature per plant and showed average temperature increase in all plants undergoing drought treatment (Figure C2). At the beginning of the experiment, only the *ca1ca4* double carbonic anhydrase knockout had a significantly lower temperature than the Col-0 control (Figure

C3.A). Over the length of the treatment, the differences in average temperature increased over most lines with the *ca1ca4* knockout maintaining a lower average temperature as compared to the Col-0 control (C3.B). The *ca1ca4* double knockout mutants maintained a constant low temperature under both watering regimes. By day 9, all plants undergoing the drought treatment were very wilted, so the temperature data may be less reliable at that late stage of soil drydown.

Plant material was collected for any plants which survived the drydown regime. An average increase in root fresh weight was observed in the CA overexpression line 4-1 both as compared to the well-watered Col-0 plants, and in comparison to the well-watered CA4-1. (Figure C.4A). Rehydrating fresh biomass to a fully turgid state, which was not significantly different than fresh weight, demonstrated that the plants were well-hydrated at the time of harvest (Figure C4.B). There was no appreciable increase in shoot mass to accompany this increase in root mass and no significant difference in shoot mass seen between lines or treatments (Figure C4). Biomass measurements were collected from a single experiment with between 3-7 plants collected per line.

Discussion

The protocol using the Profile clay soil ("Profile Porous Ceramic (PPC) "Greens Grade" soil, Profile Products LLC, Buffalo Grove, IL) for rapid soil drydown and soil moisture measured using a soil moisture was shown to be an effective method for obtaining a rapid drought treatment with uniform water loss from the soil (Figure C1). In this experiment, seeds were germinated on MS plates then transferred as seedlings

onto the fritted clay soil. An issue with this protocol is high plant morbidity at the seedling stage. Some CA-overexpression lines had many seedlings die after being transferred to the clay soil, leading to lower sample sizes for these lines. This method may be used as a rapid test to identify plant variants that demonstrate a drought-responsive leaf temperature phenotype.

The porous inorganic soil was easily removed from the root mass, allowing for simple collection of root material. Using this method for a drought-stress and recovery protocol may be useful for identifying lines such as CA4-1 that demonstrated increased root mass under drought-stressed conditions (Figure C4.A).

Methods

Germination

Arabidopsis seeds were surface sterilized with 70% ethanol, then suspended in 0.1% agar in 1.5 ml Eppendorf tubes. Seeds were pipetted onto sterile ½-strength MS-agar plates, which were then wrapped with foil and kept at 4°C for 3 days. Seeds were germinated in a growth chamber at a controlled temperature (22°C) and humidity (50 ± 2% RH) with a 16-h light:8-h dark regime at 100 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD).

Drydown soil preparation

Plants were grown in 2 inch square pots lined with polyester fabric at the bottom inside of each pot to encourage wicking and to prevent soil escape. Pots were filled with

pre-moistened “Profile Porous Ceramic (PPC) “Greens Grade” soil (Profile Products LLC, Buffalo Grove, IL) to the top of the pot. To remove dust and any possible salts from the clay, the bottom of each tray was filled with water to 2 cm up the height of the pots. The trays covered with transparent domes and allowed to soak overnight. The following day, remaining water was siphoned off from the trays and refilled with fresh water. This was repeated for a total of three times. Flats were then filled with ½ strength Hoagland’s nutrient solution, covered with domes and allowed to soak overnight. The next day, remaining standing solution was siphoned off, and the seedlings were transplanted into the pots containing saturated clay soil.

Plant growth conditions

Seedlings were transplanted one week after germination into the prepared pots and grown in a walk-in in a walk-in growth room at a controlled temperature (22°C) and humidity (60 ± 2% RH) with a 16-h light:8-h dark regime at 100 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD). Trays were bottom watered to saturation every other day, allowed to stand in water for one hour, then remaining water was siphoned off to allow oxygenation of the soil. Plants were fertilized with half-strength Hoagland’s once a week in place of watering, following the same procedure as used for watering.

Drydown regime protocol

Six weeks post-germination, pots were randomly arranged in separate trays according to watering treatment. Equal quantities of each line were arranged per tray. For the well-watered treatment, all pots were bottom watered every two days as described above. For the drought treatment, plants were not watered from days 0-10 of the treatment. The drought treatment was ended when plants had begun to wilt. After thermal imaging and soil moisture were measured on day 10 of treatment, both treatments were watered to saturation for recovery. Leaf temperature and soil moisture measurements were taken eleven days after the start of rewatering for comparison.

Infrared thermal imaging and soil moisture measurements

Using an infrared thermal imaging camera (FLIR A320, FLIR Systems Inc., Wilsonville, OR), images of whole rosette per pot were taken daily, to correlate with the drought experiment. Leaf temperature was calculated, per plant, with the Thermovision ExaminIr software (FLIR Systems Inc.). Soil moisture per pot was measured using a custom-calibrated EC-5 soil moisture probe (Decagon Devices, Pullman, WA) after thermal images were taken of each tray. A water saturated piece of filter paper was included in each thermal measurement image as a comparison.

Plant material harvest

Following leaf temperature measurements after the 11-day rewatering and recovery, plant biomass was collected. Any flower stalks were removed, and rosettes were separated from roots at the root-shoot junction. Rosettes were weighed

immediately following excision, then placed into a closed dish with the cut area submerged in water. After one hour of re-hydration, excess water was blotted from the rosettes, and turgid mass was measured. Each rosette was placed in an individually labeled bag and transferred to a drying oven. Roots were rinsed thoroughly with water to remove any remaining soil. Excess water was blotted from roots with a KimWipe, and the fresh mass was recorded. The root system from each plant was placed in an individually labeled bag and all were transferred to a drying oven. After drying for two weeks, the dry mass of each rosette and root system was recorded.

REFERENCES

Belostotsky, D. A. and Rose, A. B. (2005) 'Plant gene expression in the age of systems biology: Integrating transcriptional and post-transcriptional events', Trends in Plant Science, 10(7), pp. 347–353. doi: 10.1016/j.tplants.2005.05.004.

Berger, B., Parent, B. and Tester, M. (2010) 'High-throughput shoot imaging to study drought responses', Journal of Experimental Botany, 61(13), pp. 3519–3528. doi: 10.1093/jxb/erq201.

Hu, H., Boisson-Dernier, A., Israelsson-Nordström, M., Böhmer, M., Xue, S., Ries, A., Godoski, J., Kuhn, J. M. and Schroeder, J. I. (2010) 'Carbonic anhydrases are upstream regulators of CO2-controlled stomatal movements in guard cells.', Nature cell biology, 12(1), pp. 87-93–18. doi: 10.1038/ncb2009.

Merlot, S., Mustilli, A.-C., Genty, B., North, H., Lefebvre, V., Sotta, B., Vavasseur, A. and Giraudat, J. (2002) 'Use of infrared thermal imaging to isolate Arabidopsis mutants defective in stomatal regulation.', The Plant Journal, 30, pp. 601–9. doi: 10.1046/j.1365-313X.2002.01322.x.

Morandini, P. and Salamini, F. (2003) 'Plant biotechnology and breeding: Allied for years to come', Trends in Plant Science, 8(2), pp. 70–75. doi: 10.1016/S1360-1385(02)00027-4.

Verslues, P. E., Agarwal, M., Katiyar-Agarwal, S., Zhu, J. and Zhu, J. K. (2006) 'Methods and concepts in quantifying resistance to drought, salt and freezing, abiotic stresses that affect plant water status', The Plant Journal, 45(4), pp. 523–539. doi: 10.1111/j.1365-313X.2005.02593.x.

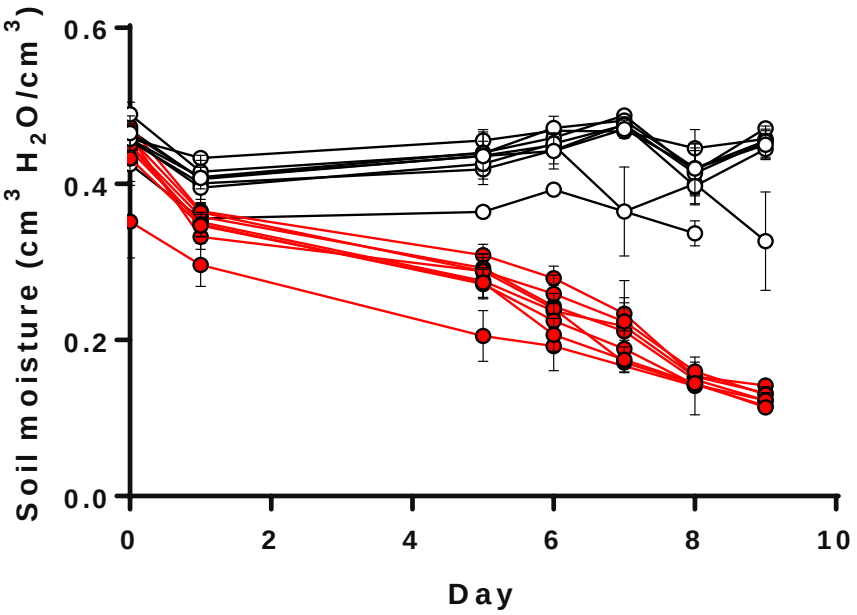

Figure C1. Soil moisture loss by treatment. On day 0, plants were 5 weeks post-germination. Pots were either rewatered to capacity (white circles) or allowed to dry without watering (red circles). Soil moisture was measured using a Decagon EC-5 soil moisture probe with a custom calibration for the Profile clay soil. The custom calibration was calculated at Decagon Devices (Pullman, WA) to relate the dielectric constant of the soil to the volumetric water content. The moisture release properties of the soil allowed for a uniform soil drydown over time.

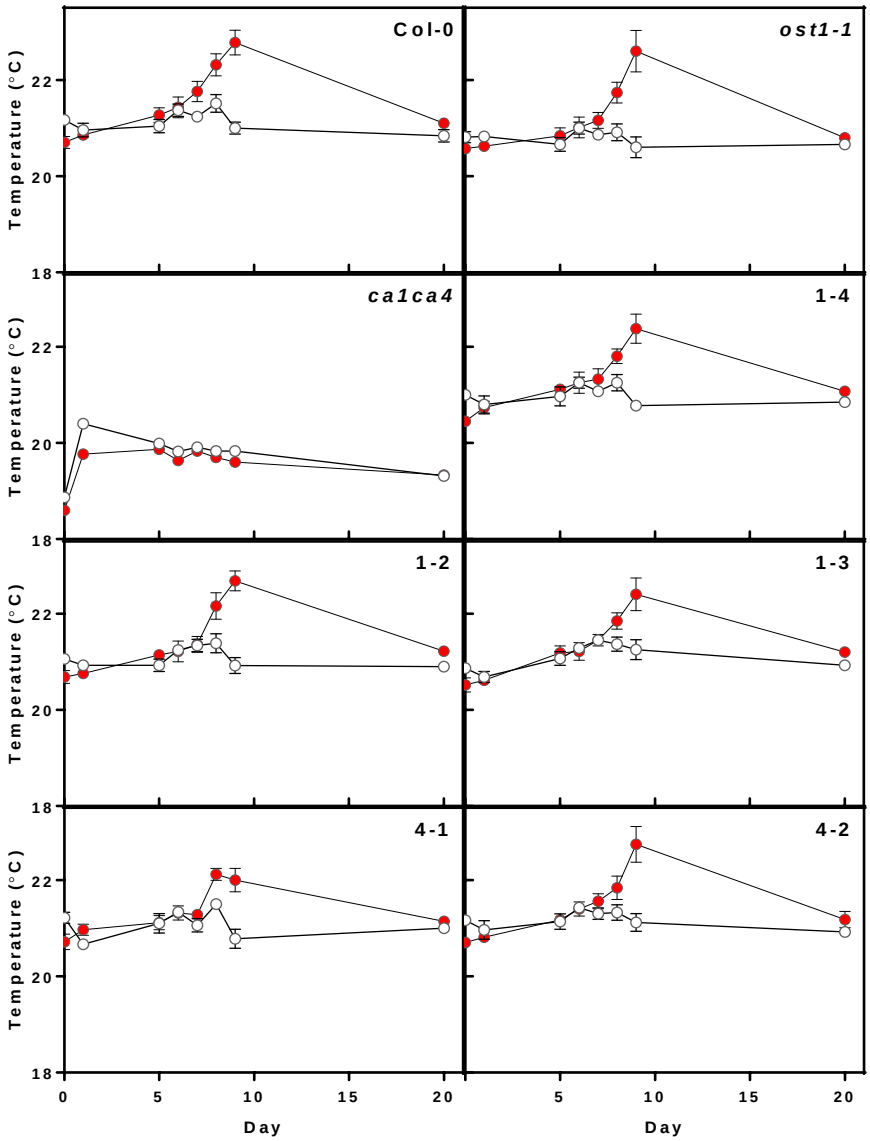

Figure C2. Average leaf temperature Plants were well-watered (white circles) or subjected to a drydown treatment (red circles) plants (means ± s.e.m.; n=3 to 6 plants). Plants were re-watered to full saturation on day 10 after all measurements were taken for the day. Day 20 measurements were taken after 11 days of watering and recovery.

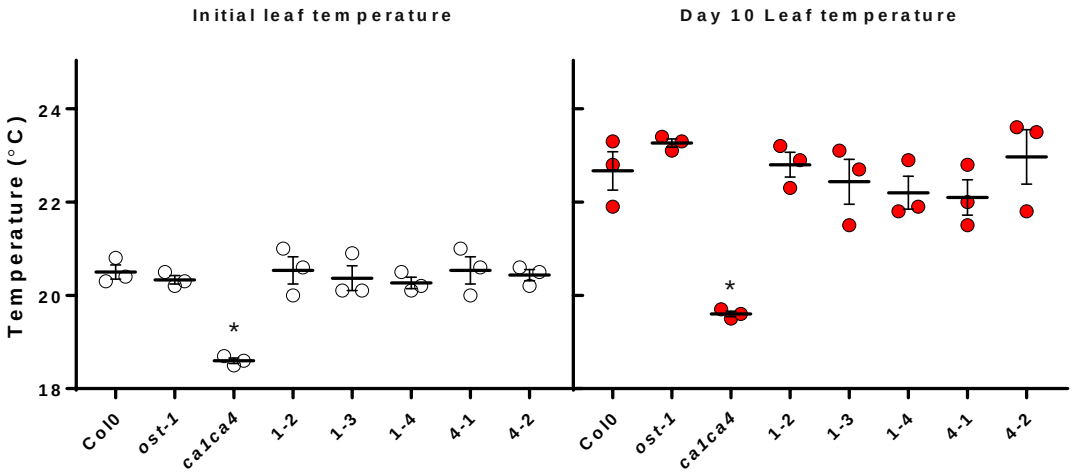

Figure C3. Average leaf temperature at beginning and end of drydown protocol. Average leaf temperature on A) first day of protocol with pots watered to soil capacity and B) following 10 days without watering (means ± s.e.m., n=3). The *ca1ca4* double carbonic anhydrase knockout had a significantly different temperature than the Col-0 control throughout the experiment. Statistical values for differences as compared to Col-0 control were calculated using a two-way ANOVA followed by Sidak's multiple comparisons test.

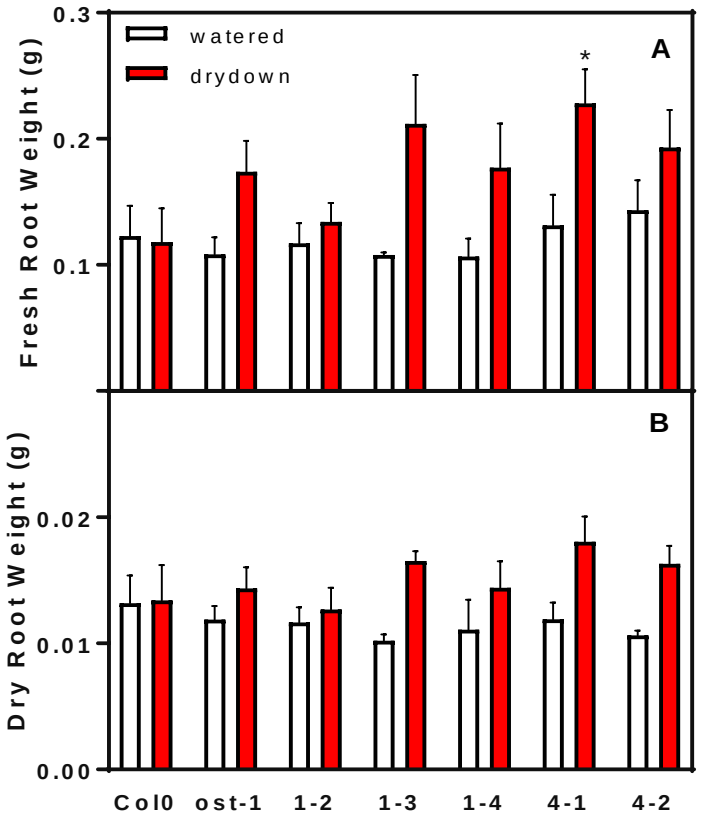

Figure C4. Harvested root mass. A) Fresh and **B)** dry root mass under watered (white bars) and drydown treatment (red bars). The fresh root mass of carbonic anhydrase knockout line 4-1 was significantly different than Col-0 plants. Plants of both conditions were re-watered to full saturation on day 10 after all measurements were taken for the day. Plant mass for both conditions was harvested after 11 days of rewatering and recovery. (Error bars denote s.e.m., n=3 to 6 plants per line). Statistical values for differences as compared to control (Watered Col-0) were calculated using a two-way ANOVA followed by Sidak's multiple comparisons test.

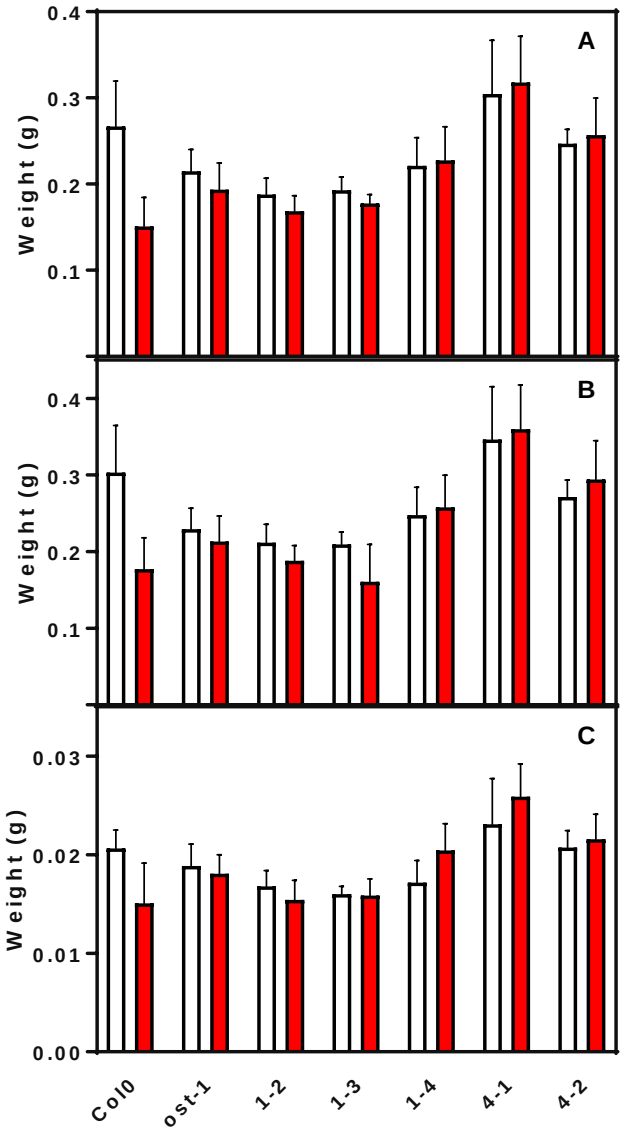

Figure C5. Harvested shoot mass. A) Fresh, **B)** turgid, and **C)** dry shoot mass under watered (white bars) and drydown treatment (red bars). Plants were re-watered to full saturation on day 10 after all measurements were taken for the day. Plant mass was harvested after 11 days of watering and recovery. Turgid mass (B) is the fresh mass following one hour of rehydration. (Error bars denote s.e.m., n=3 to 6 plants per line). Statistical values for differences as compared to control (Watered Col-0) were calculated using a two-way ANOVA followed by Sidak's multiple comparisons test.

Conclusion

This work focused primarily on understanding the mechanisms that drive differences in water use efficiency and drought tolerance in the crop plant *Brassica napus.* Since "drought tolerance" is a broad term describing the cumulative effects of numerous plant responses, it remains difficult to translate single gene effects into whole plant or crop traits that are useful for breeding. Instead, researchers can identify useful quantitative phenotypes, and use those phenotypes to identify gene targets for molecular assisted breeding.

The results described here illustrate how integrated water use efficiency measurements, in the form of stable carbon isotope data, can be used to identify potential candidates for increased water use efficiency and photosynthetic capacity. These experiments also support the use of the genetic diversity found in available crop accessions, wild relatives, and land races to identify genes linked to useful agronomic traits.

We were able to identify an interesting candidate from the *B. napus* diversity set (G302, Mozart) which demonstrated improved photosynthetic capacity corresponding to improved water use efficiency in the field. This accession can be further studied to determine the mechanisms and genes may be responsible its improved performance. It is possible that this accession may employ improved photosynthetic CO_2 uptake or fixation. Identification of quantitative trait loci (QTL) associated with the improved assimilation in this accession may help identify what genes are involved. Further, researchers are increasingly interested in the contribution of mesophyll conductance

and epidermal permeability to both water use efficiency and photosynthetic assimilation. Plants with improved mesophyll conductance may be able to maintain their photosynthetic capacity while reducing stomatal conductance under stressed conditions. Recent research has also investigated the influence of cuticle wax quantity and composition on epidermal permeability to both water and CO_2. Analysis of the wax content and composition of these plants may provide additional insight to differences in their water use efficiency.

We were also able to demonstrate a protocol inducing a controlled soil moisture deficit on plants. This method can be used to identify candidate plants that show differences in leaf temperature throughout various lengths and intensities of drought stress. This method can also be used to quantify root and shoot mass following drought stress. In the future, this method may also be useful for imposing other osmotic stresses on larger plants, such as salt stress, which may be unevenly distributed in an organic soil.

We also introduced a project that utilizes RNAi technology to target multiple PP2Cs involved in the ABA pathway. Our preliminary results from this experiment suggest that the chosen region of homology may have been similar to too many related proteins, creating multiple off-target effects such as those seen in the transformed *Arabidopsis*. Future plans include testing the transformed *B. napus* to determine if there is increased drought tolerance and/or stomatal responsiveness in plants expressing the RNAi construct driven by the drought-inducible *rd29a* promoter. We also plan to

quantify protein expression using RT-PCR to determine the effectiveness of the RNAi knockdown on expressed PP2Cs.

In summary, these projects introduce new protocols for inducing drought stress in the lab, and also for testing the effects of exogenous ABA on stomatal responses. Further, we were able to identify a strong candidate from a diverse set of *B. napus* accessions which demonstrated high water use efficiency in the field as well as increased photosynthetic capacity under lab conditions. These results provide support for translating time-integrated carbon isotope data to specific traits and mechanisms that can be targeted by breeders.