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SID 5　Research Project Final Report

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Project identification

1. Defra Project code ___ HH3608TX

2. Project title

   > Identification of genetic markers for water-use-efficiency in horticultural crops.

3. Contractor organisation(s)

   > Warwick-HRI
   > University of Warwick
   > Wellesbourne
   > Warwick
   > CV35 9EF

4. Total Defra project costs (agreed fixed price) ___ £

5. Project: start date .... 01 January 2004

   end date .... 31 December 2008

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■ Executive Summary ━━━━━━━━━━━━━━━━━━━━━━━━━━━

7. The executive summary must not exceed 2 sides in total of A4 and should be understandable to the intelligent non-scientist. It should cover the main objectives, methods and findings of the research, together with any other significant events and options for new work.

Background

Crop yield and quality are often limited by water availability, and, in many horticultural field crops, yield and quality gains make irrigation a practical and economic option. Irrigated land areas for vegetables and potatoes have risen steadily by 2.9-fold between 1982 and 2001, and the volume of water applied has risen by 4.7-fold. However, the cost and availability of water for agriculture is likely to become increasingly restrictive in the coming years due to the implementation of the Water Bill (Feb 2003), and the resulting competition for water with industrial and domestic users. In addition, scenarios of climate change in the UK, due to greenhouse gas emissions, indicate that winters will become wetter, but that summers will become drier. By 2020 in the south and east it is predicted that average summer precipitation will decrease by 20%, and that year-to-year variability will mean that on average one-in-ten summers will be considered very dry, such as the summer of 1995. By 2080, summer rainfall may have decreased by up to 50%, and soil water by up to 40%. If these scenarios are realized there would be a massive impact on agricultural practices and the rural economy in the south and east. Without extra irrigation, crop yields could only be maintained by more efficient irrigation systems, or by selecting crops cultivars that are themselves more efficient in their use of water. Water use efficiency (WUE) of a crop can be considered as the ratio between its yield and the amount of water applied to the field, or consumed by the crop. Where water rather than land area is limiting, crops with a higher WUE will produce a higher yield with the same water input, or the same yield with a lower water input. One strategy to adapt to the future predictions of lower water availability is to maximise crop WUE through breeding.

Objectives

01: Development of methodologies for measurement of WUE

Leaf carbon isotope composition (δ^{13}C) is theoretically related to leaf WUE, and leaf oxygen isotope composition (δ^{18}O) is a surrogate measure of transpiration. CO_2 assimilation rate divided by stomatal conductance (A/g_s), an instantaneous measure of WUE, can also be measured with gas-exchange equipment, or whole plant WUE can be measured by pot weighing (WUE_p). Our approach was to measure these traits, and some related component traits, in Brassica and potato field trials, and in controlled environment experiments with Arabidopsis. The aim was to define experimental systems and selection protocols for assessing WUE.

- In Arabidopsis we used three methods of measuring WUE (δ^{13}C, A/g_s and WUE_p) and found them to be in general agreement. In potato field experiments, δ^{13}C, A/g_s were also well correlated. This supports the view that δ^{13}C can be relied on as a measure of WUE in the field. This technique has the advantage of allowing large numbers of samples to be analysed, and of measuring WUE over the whole developmental period of a leaf.

- We have defined field conditions and irrigation treatments that are suitable for detecting genotype differences in WUE for *Brassica* and potatoes as measured by δ^{13}C and IRGA.

- We have demonstrated how the selection of plants with high δ^{13}C to give high WUE, can be combined with selection for low δ^{18}O and/or high young-plant biomass to ensure that breeders select for both high

WUE and high yield potential (it is known that selection for high $\delta^{13}C$ alone can sometimes lead to selection of slower growing plants).

- In several field experiments of *Brassica* and potato we observed that mild water deficits induce physiological responses that increase WUE but have little effect on yield. This supports the idea that high WUE and high yield are not mutually exclusive, and that breeding for these combined traits is an attainable goal in these species.
- We have shown that $\delta^{13}C$ is a highly sensitive measure of crop response to mild soil water deficit, and based on this we have proposed that selecting genotypes whose $\delta^{13}C$ shows a minimal response to imposed soil water deficits is an attractive alternative strategy to select genotypes that avoid water stress (e.g. by foraging for water at depth).

02: Assessment of genetic diversity of WUE
Our approach was to grow collections of genotypes and commercial cultivars under different irrigation treatments, and then to assess the range of WUE using the approaches developed in objective 01.

- We have described the genetic diversity in WUE, and related traits, in three species: in potato, genotype means for $\delta^{13}C$ across 20 genotypes fell in the range -27.75 to -25.9 ‰. For 107 *Brassica oleracea* genotypes this range was -28.3 to -25.5 ‰. For 96 *Arabidopsis* genotypes this range was from -31.9 to -29.5 ‰, and we determined that the most efficient genotype had 80% greater WUE than the least efficient genotype as measured by dry weight gain per unit of water transpired.
- Genetic manipulation to increase ABA content in Arabidopsis genotype Col-0 led to a 56% improvement in WUE, and the maximum WUE achieved by this method was greater than that of any natural accession.

03: Identification and resolution of robust WUE QTLs
We assessed WUE using $\delta^{13}C$ in mapping populations of *Brassica oleracea* in the field, and of *Arabidopsis* in controlled environments. Data analysis allowed the locations of genes that influence WUE to be mapped.

- We have identified five quantitative genetic loci (QTL) in *Arabidopsis* that control WUE; four of these are supported by both $\delta^{13}C$ and gravimetric (WUE_p) data, and three are not previously reported.
- In *Brassica oleracea* we have identified six QTL for $\delta^{13}C$, and we have highlighted three regions (C7, C8, C9) where QTL are reproducible between transplantings or sites.
- In addition to $\delta^{13}C$ data, we have collected biomass, $\delta^{18}O$, SPAD and specific leaf weight data (plus WUE_p for *Arabidopsis*). Co-localising QTL for these traits provides clues about the physiological mechanisms for the control of $\delta^{13}C$.

04: Validation of QTLs

- In *Arabidopsis,* we have begun to construct near-isogenic lines in the region of three QTL detected in the Col-*gl*1 x Kas-1 population. We have data supporting the existence of two WUE_p QTL after two rounds of backcrossing.
- In *Arabidopsis,* we have used association mapping in 96 accessions to detect loci within the Col-gl1 x Kas-1 QTL that are associated with WUE_p and $\delta^{13}C$; on this basis we have highlighted genes close to those markers that might be causative.
- In *Brassica oleracea* we highlighted a region of C7 that controls WUE based on both A12 x GD33 and Nedcha x Gower mapping populations. We have validated this QTL by assessing substitution lines in field and glass experiments. Physiological assessment of line SL118 carrying a substitution on C7 shows a 17% effects on g DW kg^{-1} H$_2$0, driven by changes in stomatal conductance.
- The QTL on C9 detected in the A12 x GD33 *Brassica* population was validated by performing association analysis; we discovered that a marker within this QTL that was associated with $\delta^{13}C$.

Application of QTL for WUE in plant breeding
Our genetic analysis could be applied to breeding for WUE provided that further fine mapping of QTL can be achieved so that tightly linked markers can be offered to breeding companies. Such markers could be used for MAS to ensure that beneficial alleles for WUE were maintained in breeding programs while phenotypic selections for unrelated traits were made. This would raise the baseline for WUE in breeding populations. By combining markers for several key WUE QTL, incremental increases in WUE should be achievable.

Options for new work
We are fine mapping Arabidopsis QTL for WUE in project WU0116 by backcrossing and screening for recombinants, and we are developing improvements in genome-wide association mapping in *Arabidopsis* (where 240,000 single nucleotide polymorphism (SNP) loci have been recently made publicly available for such studies). Once the underlying genes are identified in Arabidopsis, we can test their utility in field crops. We are also fine mapping the QTL for WUE on C7 in *Brassica oleracee*

This report contains the first attempts at association mapping in *Brassica oleracea* and the strong QTL for $\delta^{13}C$ on chromosome C9 was confirmed by our association mapping data. This provides a target region that would be ideal for more refined mapping and then allele testing. In the future we will need to increase the density of molecular markers in the target regions by employing high throughput SNP platforms; this will allow fine mapping of QTL and is required to find more closely linked markers suitable for marker-assisted selection (MAS).

Data from this project could be applied to other *Brassica* crops, particularly broad acre oilseed crops where WUE and drought tolerance are of great interest globally. For example, once fine mapping of QTL is more advanced, we could perform targeted association analysis, and construct near-isogenic lines in *Brassica napus* for testing in the field.

Our work in transgenic tomato has shown that single transgenes can have dramatic effects on WUE_p that can exceed the natural variation available (e.g. 80% increase compared to 17% increase for the best *Brassica* QTL), and that have little or no impact on biomass production [7]. This approach should be pursued in parallel to the exploitation of natural variation to make the most rapid advances in the breeding of high WUE crops.

In potato there is an urgent need to develop cultivars that can be productive with reduced irrigation. We have suggested two ways of selecting for such cultivars using stable isotope analysis: a screen for high WUE and high yield, and a screen for low response to soil water deficit.

■ Project Report to Defra

8. As a guide this report should be no longer than 20 sides of A4. This report is to provide Defra with details of the outputs of the research project for internal purposes; to meet the terms of the contract; and to allow Defra to publish details of the outputs to meet Environmental Information Regulation or Freedom of Information obligations. This short report to Defra does not preclude contractors from also seeking to publish a full, formal scientific report/paper in an appropriate scientific or other journal/publication. Indeed, Defra actively encourages such publications as part of the contract terms. The report to Defra should include:

- the scientific objectives as set out in the contract;
- the extent to which the objectives set out in the contract have been met;
- details of methods used and the results obtained, including statistical analysis (if appropriate);
- a discussion of the results and their reliability;
- the main implications of the findings;
- possible future work; and
- any action resulting from the research (e.g. IP, Knowledge Transfer).

1.   Introduction

This project had two main scientific aims: (i) to establish the genetic diversity of water use efficiency (WUE) in cultivars and germplasm of both vegetable Brassicas (*Brassica oleracea*) and potatoes, and (ii) to identify the heritable components of variation in WUE, and to identify and validate molecular markers for use in selection of plant varieties with improved WUE.

Three experimental systems were required for the project: for *Arabidopsis thaliana, Brassica oleracea* and potato. *B. oleracea* was used as the main model crop for the study of the genetic basis of WUE and to obtain markers applicable in the Brassicaceae. Work was also performed in *Arabidopsis* with the aims of (i) using the very advanced genetic and genomic resources in *Arabidopsis* to move more rapidly towards identification of candidate genes that might have utility in a wide range of crops, and (ii) to use *Arabidopsis-Brassica* comparative analysis to highlight genomic regions with conserved functions in WUE, and to thereby make more rapid progress in fine mapping *Brassica* QTL based on information from *Arabidopsis.*

The five objectives of the project were:

01: Development of methodologies for measurement of WUE
02: Assessment of genetic diversity of WUE
03: Identification and resolution of robust WUE QTLs
04: Validation of QTLs
05: Technology transfer

These objectives cut across the three experimental systems, and so this report is structured based on the three separate species, with each objective considered for each species. We also discuss commonalities and comparative aspects. A more detailed report is available as an appendix.

1.1.   Strategy for assessing water use efficiency in plants and crops

Growers seek the highest yield of their crops for a given level of water input as rainfall or irrigation, and regulators such as the Environment Agency demand that they can demonstrate efficient use of water. In our field experiments our strategy was to measure the **response of yield to different irrigation treatments**; an ideal genotype would be high yielding in optimum conditions and would then have a low yield response to reduced irrigation (and so would be considered *drought resistant*). In some cases we also measured **canopy cover** to estimate the response of crop growth to irrigation.

In the field experiments we measured the intrinsic WUE (**WUE$_i$**). This value is "intrinsic" because it depends on the "intrinsic" stomatal conductance (**g$_s$**) rather than on transpiration (**E**) which depends on both g$_s$ and environmental factors such as temperature and relative humidity. We measured WUE$_i$ by two approaches: (i) measurement of carbon isotope composition (**δ^{13}C**) which can be theoretically related to WUE$_i$ and (ii) by use of an infrared gas analyser (**IRGA**) that can directly measure leaf CO_2 assimilation rate (**A**) and stomatal conductance (**g$_s$**), allowing WUE$_i$ to be calculated as **A/g$_s$**. In pot-grown plants in glasshouse experiments, we also determined WUE at the whole plant level (**WUE$_p$**) using gravimetric approaches by calculating the ratio of final shoot dry weight to the mass of water lost from the pots (E) during the growth period.

Because WUE$_i$ is a ratio between *A* and *g$_s$*, its value does not inform on the values of these two components, or whether a difference in WUE$_i$ is due to a change in *A* or *g$_s$*. For this reason we also measured traits that should relate to *A* or *g$_s$* independently. *A* (a measure of CO_2 assimilation per unit leaf area) should relate to the photosynthetic capacity per area of leaf, and estimates of this are given by leaf chlorophyll content measured by a chlorophyll content meter (CCM-200, Optisciences Ltd), and by the leaf thickness (measured as leaf dry weight/leaf area, or mass per leaf area, **MLA**, also referred to as the reciprocal, specific leaf area or **SLA**). More accurate measurements of this were obtained in some cases using IRGA to determine maximum assimilation rate at saturating levels of light or CO_2. Transpiration (related to *g$_s$*) can be estimated from oxygen isotope composition (**δ^{18}O**), although the usefulness of this trait was relatively unknown at the beginning of the project.

2.   Potato (*Solanum tuberosum* L., subsp. tuberosum*)*

2.1.   Objective 01: Development of methodologies for measurement of WUE (potato)
2.1.1.   Experimental design: Kirton
Field trials at Kirton aimed at establishing a protocol for imposing water stress and measuring WUE in potato germplasm were carried out in 2005. Our target was to identify physiological parameters that correlate with the baseline measurement of WUE, i.e. the response of tuber yield to irrigation, or simply yield under water limited environments. Such physiological parameters would then be candidates for screening larger numbers of genotypes without the expense of growing crops to maturity at multiple levels of irrigation.

Ten maincrop cultivars were chosen based on a range of drought tolerance scores provided by either breeding companies or the cultivar database of the British Potato Council. The trial was restricted to maincrop cultivars to reduce effects on WUE due to developmental/phenological traits such as flowering time and haulm longevity. Chitted tubers were planted in ridges under Spanish tunnels at Kirton. Each plot consisted of ten individual seed tubers. There were 120 plots in total, representing all combinations of ten varieties and three soil moisture regimes, replicated four times over six tunnels. Irrigation was delivered by means of trickle-tape along the top of the ridges. Access tubes were installed in the ridge of each main plot and were used to take readings of soil moisture content down to a depth of 80 cm by inserting a Sentek Diviner 2000 capacitance probe into the access tubes.   During the trial the following measurements were made: Crop canopy cover (Sunfleck Ceptometer, Delta-T Devices), leaf gas exchange data (CIRAS -1 IRGA, PP systems), leaf and tuber samples were taken for δ^{13}C and δ^{18}O analysis, leaf chlorophyll content, specific leaf area (SLA, g leaf DW cm^{-2}, leaf areas determined by flat bed scanner and image analysis), tuber grades and total tuber yield and dry matter.

2.1.2.   Experimental design (ADAS)
Because of the difficulty in imposing sufficient soil moisture deficits at depth at the Kirton site in 2005 we grew 20 cultivars of potato on a lighter, sandy soil under mobile rain covers at ADAS, Gleadthorpe in 2006. We collected data on canopy cover, SLA and chlorophyll content, leaf δ^{13}C and δ^{18}O and also some preliminary data was collected for canopy temperature using thermal imaging (by Prof Lyn Jones, U. Dundee). Because the 2006 Brassica and potato trials were switched between Kirton and Gleadthorpe we had insufficient manpower at Gleadthorpe for tuber recovery in this trial, however, since it has previously been reported that canopy cover is a good estimate of final tuber yield [1], we adopted a strategy of recording canopy cover in the different irrigation treatments, and then looking for relationships with other parameters.

The twenty commercial potato cultivars were grown under four levels of irrigation: control, and then d1, d2 and d3 (increasing extent of drought treatment). Eight cultivars were in common with the ten grown at Kirton in 2005. The aims were:
•   to look for easy-to-measure traits that correlate to WUE or drought resistance (as measured by canopy development) that could be used in a breeding program
•   to establish the extent of genetic variation for traits related to WUE

2.1.3.   Development of Methodologies for potato

2.1.3.1. Measuring WUE

$\delta^{13}C$ (surrogate of WUE) and $\delta^{18}O$ (surrogate of transpiration) responded sensitively to irrigation treatment in the direction expected by theory at both field sites. There was also a good relationship between $\delta^{18}O$ and transpiration and between $\delta^{13}C$ and A/g_s when comparing genotypes. Importantly, there was very close agreement in the ranking of cultivars for $\delta^{13}C$ at the two sites, suggesting that this trait has a low genetic x environment interaction, or in other words if selections were made in one location, the varieties would maintain their WUE behaviour in a range of environments.
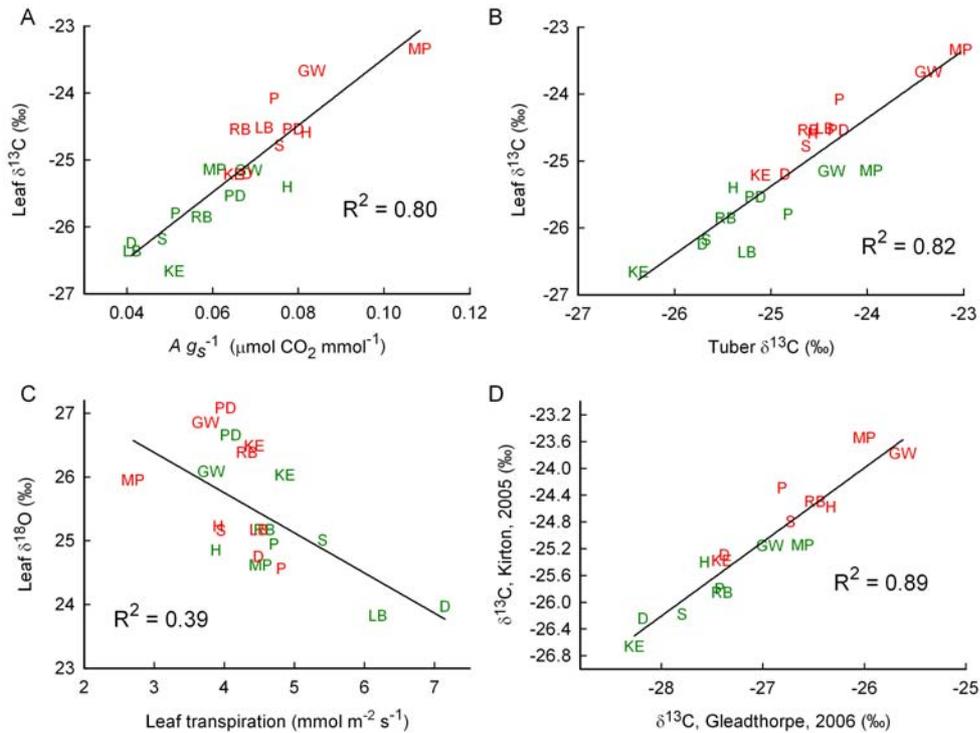
Figure 1: Validity and robustness of stable isotope data. A: $\delta^{13}C$ as a measure of intrinsic water use efficiency (WUE$_i$) - $\delta^{13}C$ is plotted against WUE$_i$ as measured by infrared gas analyser ($A\ g_s^{-1}$; ratio of CO_2 assimilation rate to stomatal conductance). B: correlation between leaf and tuber $\delta^{13}C$ (Kirton, 2005). C: $\delta^{18}O$ as a measure of transpiration rate - $\delta^{18}O$ is plotted against the transpiration measured by IRGA (Kirton, 2005). D: Robustness of $\delta^{13}C$ between sites (Kirton, 2005 against Gleadthorpe, 2006). For A, B and C means for each cultivar (n = 4) are shown for the control (green letters) and dry2 (red letters) treatments. For D, only the eight cultivars that were common between the two trials are shown. Data points are represented as letters for cultivars: D, Desiree; GW, Golden Wonder; H, Horizon; KE, King Edward; LB, Lady Balfour; MP, Maris Piper; PD, Pentland Dell; P, Picasso; RB, Russet Burbank; S, Saturna.
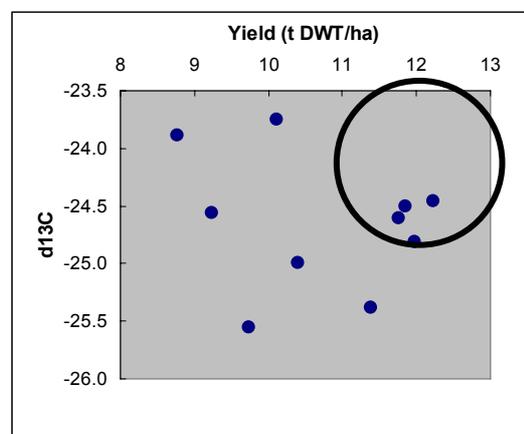

Figure 2: Selection of genotypes based on $\delta^{13}C$ and yield. Data from the dry1 treatment are plotted from Kirton 2005 (similar results obtained from treatment means). The circle indicates the zone of high yield and high WUE. The four genotyes within are Picasso, Pentland Dell, Saturna, Russet Burbank.

2.1.3.2. *Relationship between yield response and WUE*

Cultivar improvements in WUE, to be useful to UK growers, must give the same or higher yields with reduced water inputs. Our rationale is that selecting for improved WUE either at the leaf or field level (see above), can help to maintain yield as water inputs go down. Our experiments were designed to test this rationale by correlating WUE and yield response.

In the Kirton trial we failed to generate sufficient soil water deficit at depth and so the tuber yield reduction was only 12% in the driest treatment. It is clear that the choice of field site and the soil hydrological properties are critical in designing a breeding screen - i.e. to generate useful treatment effects the initial available soil water must be much less than expected seasonal evapotranspiration, and upward water movement from the water table must be minimal. The Kirton soil limited the power of the experiment to find correlations between response of tuber yield and the physiological parameters measured. At Gleadthorpe we were able to generate larger soil water deficits, and big differences in canopy formation were observed, but again correlations to other parameters were poor. The lack of correlation could reflect too narrow a range of values due to lack of genetic or treatment variation, or large confounding environmental variability. Alternatively, the complex nature of the yield response trait may mean that so many different independent variables are important that correlations with single traits are low. Literature indicates that several other studies have failed to find strong correlations. Because of the lack of correlation there remains some uncertainty about how $\delta^{13}C$ would influence water productivity at the crop scale if it was used as a basis for selection in breeding programs. However, based on theoretical considerations, we have suggested two strategies for the use of $\delta^{13}C$ as a selection tool in breeding programs (see section 2.2.2. Modelling approaches may help to strengthen the confidence that selection based on $\delta^{13}C$ behaviour will lead to maintained or improved tuber yield with lower water inputs.

One concern is that selection for high WUE alone would lead to depression of yield potential because low transpiration would limit CO_2 uptake. However, our experiments provide good evidence that WUE can be increased substantially with very little impact on yield. This conclusion arises from the observations of large physiological changes that result in lower water use in our dry treatments at both sites despite little or no change in canopy or tuber yield. **These observations support the notion that either regulated deficit irrigation, or partial root drying treatments, or indeed plant breeding approaches, could succeed as a means to improve WUE of potato without dramatic yield losses.**

2.2.   Objective 02: Assessment of genetic diversity in potato
2.2.1.   *Genetic Diversity of WUE in potato*

In the Kirton trial there was no evidence that the different cultivars responded differently to the irrigation treatments for any parameter, and this is probably at least partly explained by the small treatment effects. However, there was good evidence for differences in WUE between cultivars irrespective of treatment. Both $\delta^{13}C$ and $\delta^{18}O$ were significantly different between cultivars, and this alone could be used to rank cultivars according to WUE_i and transpiration. The range of $\delta^{13}C$ values observed in the Gleadthorpe experiment was -27.75 to -25.9 ‰. Of the ten cultivars grown, Maris Piper and Golden Wonder had the highest $\delta^{13}C$ values (high WUE_i). Maris Piper had an intermediate leaf $\delta^{18}O$ value suggesting that it was not abnormally low transpiration that drove the high WUE, and it also gave the smallest yield reduction in the dry treatments although this was not significantly different to other cultivars. Maris Piper is a high yielding, popular commercial cultivar, but we note that it is given a relatively low drought tolerance rating of 4 out of 10 by the BPC. The scientific basis for this score is not clear.

For $\delta^{18}O$ there was an interesting interaction between tissue type and genotype; for most genotypes, $\delta^{18}O$ values were higher in the tuber, but for Pentland Dell $\delta^{18}O$ values were significantly higher in leaf than tuber, although the physiological basis for this is not known.

At ADAS where larger soil water deficits were achieved, there were significant genotype x treatment interactions for both $\delta^{18}O$ and $\delta^{13}C$, showing that genotypes were responding differently to the drought treatments in their leaf physiology, even though we could not detect differences in the response of their canopy development. There were genotypes that had atypical responses of $\delta^{13}C$ and $\delta^{18}O$, for example most genotypes responded to the driest treatment by increasing $\delta^{13}C$ and $\delta^{18}O$, suggesting that increases in WUE were occurring as driven by reduced transpiration (theory dictates that $\delta^{13}C$ is positively correlated with WUE and that $\delta^{18}O$ is negatively correlated to transpiration). However "Golden Wonder" was unusual in that it had high $\delta^{18}O$ in control conditions, and values did not increase during drought, whereas it did show the typical increase in $\delta^{13}C$ (and so WUE). This suggests that "Golden Wonder" was able to increase WUE by improving assimilation rate per leaf area, rather than reducing g_s. In the case of "Hermes", the driest treatment induced hardly any change in WUE, but there was still the typical increase in $\delta^{18}O$ (reduced transpiration), which therefore may have been accompanied by a proportionate decrease in assimilation. "Horizon" had significantly higher g_s than other genotypes as measured by IRGA, but WUE was not different. Leaf temperature (infrared thermography, Prof. Lyn Jones) and $\delta^{18}O$ were in general agreement with the higher g_s of "Horizon".

2.2.2.   *Conclusions from Potato work*

Objective 01: Correlations between A/gs and $\delta^{13}C$, and between transpiration and $\delta^{18}O$ support the idea that these stable isotope analyses do give a good measure of leaf level WUE and transpiration, and that they will be useful selection tool in breeding programs. However, correlations between leaf level WUE (or related parameters) and yield response to water deficit could not be determined in our experiments. Crop modelling approaches may be the best strategy to predict the effects of selecting for a particular trait on yield under water deficit.

We propose that stable isotope analysis could be used in two contrasting ways in a potato breeding program:

(i) to select lines that have a small response of $\delta^{13}C$ to soil water deficit; this would indicate that the line has increased use of the available soil water, and so avoids shoot water deficit. This may come about by, for example, deeper rooting, or by a reduced physiological response to the mild soil drying that reduces growth and productivity. Selected genotypes would maximise their use of available water, and maintain productivity as soil water declines. They would have lower leaf WUE, but would give a higher yield per unit of water applied to the field (higher field level WUE). Potentially, SPAD and MLA could be used in the same way. This strategy would suit an environment with an unpredictable rain fall pattern – the crop would continue to grow through short dry spells, and then soil water would be replenished.

(ii) to select lines that have high $\delta^{13}C$ (high WUE), but also high yield as measured by canopy development, tuber yield or possibly low $\delta^{18}O$ (i.e. high transpiration which is associated with high yield). This would provide genotypes that have high WUE, but not at the expense of good yield potential (e.g. Figure 2). They are likely to arise from increased A rather than decreased g_s. This strategy is better suited to environments where the crop grows on stored soil water, with long intervals with no rainfall. The slower use of soil water delays the onset of drought stress.

Objective 02: Although commercial potato cultivars have a narrow genetic base, we were able to detect genetic variation in WUE. $\delta^{13}C$ and $\delta^{18}O$ showed significant cultivar differences, and so cultivars could be ranked on this basis. Maris Piper, one of the most popular cultivars in UK production, was ranked highest based on $\delta^{13}C$. Other cultivars (Golden Wonder, Hermes, Pentland Dell) had patterns of $\delta^{13}C$ and $\delta^{18}O$ that were significantly different from other cultivars, suggesting that they had different water use physiology.

3. Arabidopsis
3.1. Objective 01: Development of methodologies for measurement of WUE (*Arabidopsis*)

Preliminary experiments to identify an optimal experimental system were carried out by growing four *Arabidopsis* accessions (Col, Cvi, Kas and Ler) in a range of containers, media and controlled environment conditions. John Innes no. 2 compost in 50 ml falcon tubes arranged in racks, purpose built to fit the cabinets (Figure 3) provided the best growing conditions. The racks provide a dark environment for the transparent tubes such that root growth can be monitored by removing individual tubes from the rack. The environmental variables tested were VPD (Vapour pressure deficit, a measure of the ability of the atmosphere to remove water from the plant based on temperature and relative humidity gradients), soil moisture content and day-length. Based on these experiments, VPD and soil water content parameters were chosen for further larger experiments with diversity sets and mapping populations (full experimental details are available[2]).

Single plant gas-exchange chambers (Figure 3) and small leaf chambers designed for *Arabidopsis* work were purchased and protocols developed for measuring transpiration and assimilation of single *Arabidopsis* plants or leaves using a CIRAS infra-red gas analyser. These methods were found appropriate for the study of small numbers of selected genotypes.
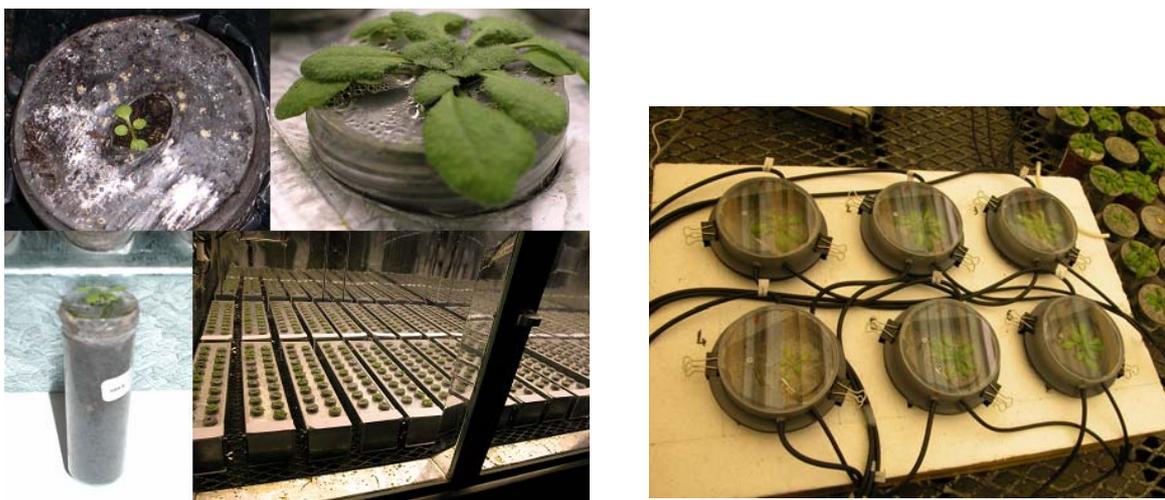

Figure 3: Growing system for *Arabidopsis* in controlled environment cabinet. Left panel: Clockwise from top left: rosette stage 1.02, when gravimetric measurements start; rosette development above a Parafilm layer; *A. thaliana* seedling growing in a 50 ml centrifuge tube; aluminium racks set-up in the Saxcil controlled environment cabinet. Tube diameter: 28 mm. **Right pane: Set of six whole-plant gas-exchange chambers.**
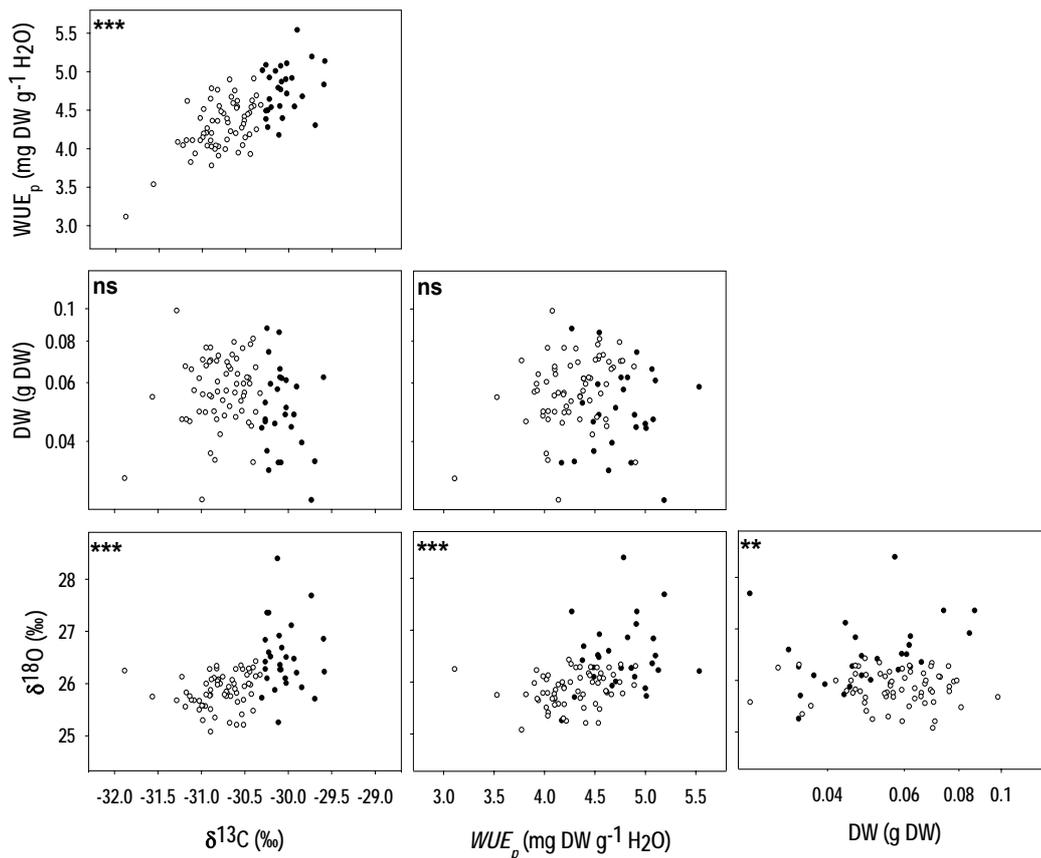
Figure 4: Scatter plot matrix between accession means (means between treatments and replicate experiments). Open circles represent accessions with $\delta^{13}C$ < -30.3 ‰ and closed circles represent accessions with $\delta^{13}C$ ≥ -30.3 ‰. Taken from J-C Deswarte PhD Thesis. DW, dry weight of shoot; WUE_p, whole plant water use efficiency measured gravimetrically.

3.2.   Objective 02: Assessment of genetic diversity of WUE (*Arabidopsis*)

Seed were obtained from the Nottingham *Arabidopsis* Seed Center for an established diversity set consisting of 96 accessions from around the world known as the "Nordborg collection" [3]. The collection has been extensively genotyped and genome-wide polymorphism data is publicly available [4]. Seed were bulked in the glasshouse and an experiment was run under high and low VPD, at a soil moisture content that provides a mild stress treatment [2]. Measurements of shoot biomass, leaf area, root extension rate, gravimetric water use efficiency, $\delta^{18}O$ and $\delta^{13}C$, rate of drying of detached shoot, leaf thickness, and leaf SPAD (estimate of chlorophyll content/leaf area) were made. Most of the traits measured showed significant accession effects that allowed extreme accessions to be identified. Examples of the data are shown in Figure 4. Data for repeat experiments was highly correlated, showing that the assays were robust and reproducible. This trait data describes the genetic diversity (e.g. 3.1 to 5.5 mg DW per g H_2O for gravimetric WUE), and was also used for the allelic association approach (see section 3.3.1).

3.3.   Objective 03: Identification and resolution of robust WUE QTLs

Identification of water-use efficiency QTLs in *A. thaliana* focused upon the "Somerville" Col-*gl*1 x Kas-1 mapping population, consisting of 128 recombinant inbred lines (RILs). In addition, efforts to identify water-use efficiency QTL were made in the "Bancroft" Nok-3 x Ga-0 mapping population. The Col-*gl*1 x Kas-1 mapping population was assessed in two separate controlled environment cabinet experiments. A robust QTL analysis was performed using both QTL Café and WinQTL Cartographer. These well-establish methods gave consistent results for WUE and related leaf traits (Figure 5). Several clusters of QTL related to WUE were found, and four were highlighted that impact directly on WUE_p. The QTLs related to WUE_p or biomass had genetic contributions of around 15-20%. The Nok-3 x Ga-0 mapping population revealed QTL collocating with two of the three regions highlighted in the Col x Kas-1 mapping population on LG4 and 5.

We decided to focus further work on three regions of the *A. thaliana* genome, two regions on LG4 and one on LG5 (Figure 5). We instigated a programme of backcrossing and marker screening to develop a panel of near isogenic lines (NILs) in these three regions. Preliminary gravimetric experiments with NILs at two of the QTL (BC_2F_3 generation) showed effects on WUE that validated the QTL. This work is continuing in Defra project WU0116.
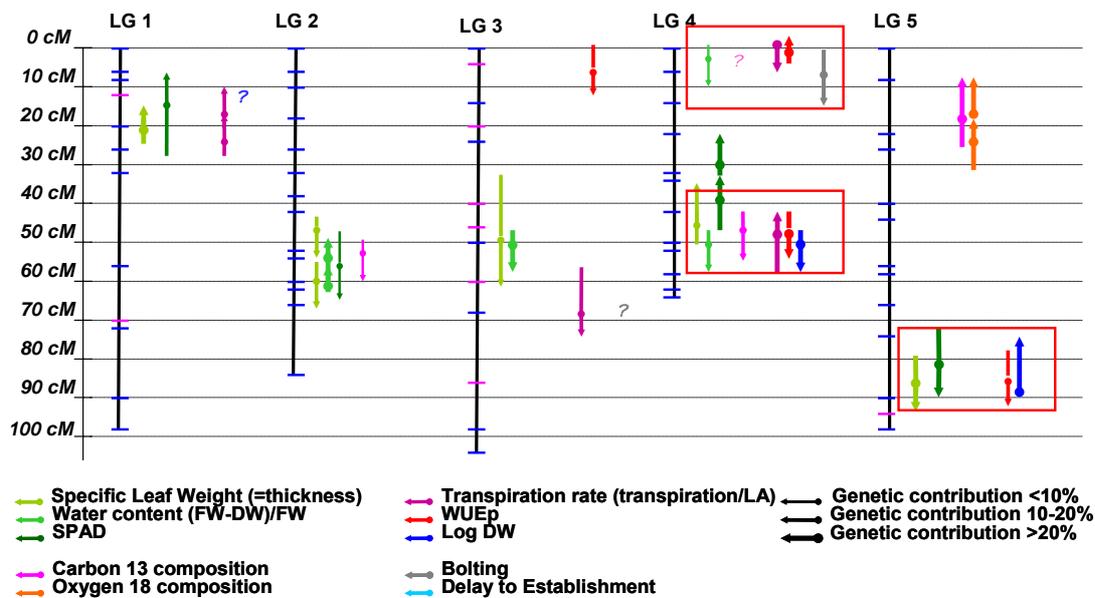
Figure 5: QTL map obtained with the Col-gl1 x Kas-1 population. Combined results of two experiments, using mean from replicated well-watered and water-limited treatments. QTL position, confidence interval and genetic contribution were estimated with the Composite Interval Mapping procedure with WinQTL Cartographer. The 3 most interesting regions are highlighted by red boxes.

3.3.1. Association mapping in Arabidopsis

This project generated physiological data relating to WUE in the *Arabidopsis* 'Nordborg' diversity set (Objective 02). In combination with publicly available genome-wide genotype data (http://walnut.usc.edu/2010/), two successive linkage disequilibrium association analyses were performed. The first analysis related phenotypic variation with allelic variation at each individual marker. The second analysis was a modification of a published method [4]. A neighbour-joining phylogeny was estimated at each locus, based on the differences between the haplotypes identified in the first analysis, and this was used to produce a series of divisions of the accessions into two groups. The divisions considered were between the accessions with each haploytype and the other accessions, and between the accessions with the haplotypes which were grouped by each internal node of the phylogeny and the other accessions. The associations were assessed using Mann-Whitney U tests, and the most significant division was used to characterise the locus.

Notwithstanding the complexities of association analysis and the probability of false positives (discussed in the appended report) it is encouraging that within each of our three selected WUE QTL domains there is at least one locus predicted to have an association with WUE$_p$. This information can be used to focus the fine mapping of the three QTL identified in the Col-gl1 x Kas-1 populations. The work can now also be extended to use 240,000 genome-wide single nucleotide polymorphisms for even higher resolution association mapping.

3.3.2. Comparison of transgenic improvement in WUE with natural variation.

We produced transgenic *Arabidopsis* lines with the aim of increasing ABA biosynthesis and these had reduced stomatal conductance and transpiration rate. They showed that affecting stomatal control was a powerful way to increase WUE$_p$ (+50%), and most of the time this had no significant, or only a small negative effect, on biomass (data not shown). In agreement with this, in the "Nordborg" diversity set, WUE$_p$ and biomass were not correlated either; this suggests that although stomatal control is a significant determinant for WUE$_p$ it is not the only trait that influences WUE in the natural accessions of *A. thaliana*, otherwise high WUE$_p$ would be expected to be associated with low g_s and generally lower biomass (due to reduced CO_2 uptake).

3.3.3. Understanding the physiological basis of WUE$_p$ in Arabidopsis

Insights into the physiological processes involved in WUE$_p$ were provided by further controlled environment cabinet experiments. Ten accessions contrasting for gravimetric WUE$_p$ and biomass were selected for further analysis. Stomatal conductance (g_s), assimilation rate (A) and intrinsic WUE (WUE$_i$ = A/g_s) were measured with gas-exchange equipment. The results show significant differences in g_s, but little differences in A. Stomatal control also influenced significantly A and WUE$_i$, and to a lesser extent WUE$_p$, but was not correlated to higher biomass (Table 1). Stable isotope ($δ^{13}C$ and $δ^{18}O$) data was also measured for these 10 selected accessions. We found that $δ^{13}C$ correlated well with WUE$_p$ and WUE$_i$, whereas the expected relationship between $δ^{18}O$ and g_s was not found. $δ^{18}O$ correlated more closely with leaf traits such as water content, specific leaf weight and leaf greenness, although the mechanistic basis of this is not clear. **In the growth conditions we imposed, stomatal conductance (g_s) appears to be the main, but not sole, factor driving the variation in WUE, but this did not correlate to biomass.**

Table 1: Correlations between instantaneous gas exchange parameters and long-term gravimetric water-use efficiency within ten contrasting accessions. Accessions were selected from the Nordborg collection. n= 68 plants; values in bold are significant. *A*, CO_2 assimilation rate; Biomass, shoot dry weight; WUE_i, A/g_s; WUE_p, gravimetric water use efficiency; g_s, stomatal conductance.

Trait	*A*	Biomass	WUE_i	WUE_p	g_s
A	1				
Biomass	0.16	1			
WUE_i	0.09	-0.27	1		
WUE_p	-0.07	0.06	**0.62**	1	
g_s	**0.65**	0.21	**-0.62**	-0.52	1

3.3.4. Conclusions from Arabidopsis work
Objective 01: Robust methods were developed in which soil type, water content and environmental conditions were defined, and $\delta^{13}C$, A/g_s and WUE_p were all found to correlate well and so can be used largely interchangeably to assess WUE in this species.

Objective 02: 96 *Arabidopsis* accessions were assessed and results were highly reproducible when comparing between different experiments. The range of WUE_p values obtained was from 3.1 to 5.5 mg DW per g H_2O. This represents significant genetic diversity in WUE, however, transgenic *Arabidopsis* plants that over-accumulate abscisic acid had better WUE_p than any naturally occurring accession in this collection.

Objective 03: Five QTL for gravimetric WUE_p were found, three were novel compared to published QTL. Some QTL co-localised with flowering time and biomass, but greater resolution is needed to distinguish between gene pleiotropy and linkage in these cases.

Objective 04: Two QTL were validated by preliminary results from near-isogenic lines produced by back-crossing (4-2 and 5-1). Further validation of the QTL regions (4-1, 4-2 and 5-1) was provided by genome-wide association mapping: all three QTL contained highly significant associations with WUE_p. The markers used are on average about 100 kilobases apart (~10-20 genes). This provides a major advance towards gene identification.

4. *Brassica oleracea* (vegetable Brassicas)
Vegetable Brassicas were chosen as the main system in which to explore the genetics of WUE in a horticultural crop. Trait assessment was performed in the field to ensure that QTL identified were relevant to field-grown crops. *Brassica oleracea* has a range of genetic resources available (mapping populations, substitution lines, genomics, and close relationship with *Arabidopsis thaliana* where the full genomic sequence is known).

4.1. Objective 01: Development of methodologies for measurement of WUE (*B. oleracea*)
4.1.1. Rationale and experimental design.
Our aim was to establish methods for determining WUE in field grown brassicas. In particular it was important to define the level of soil water deficit required to elicit a growth response, and to investigate the use of isotope discrimination analysis and biomass accumulation in young plants as agronomically useful measures of WUE. In the first year, field trials were run at Kirton and Gleadthorpe using ten genotypes. The chosen lines represented the diversity of vegetable brassica crops, but also included four doubled-haploid (DH) lines from the Nedcha x Gower (Brussels sprout x cauliflower) mapping population. The DH mapping lines had previously shown differential carbon isotope discrimination ($\Delta^{13}C$) and intrinsic water use efficiency (WUE_i), and were therefore used as controls with known genetic differences in WUE [5]. $\Delta^{13}C$ is carbon isotope discrimination and is inversely related to $\delta^{13}C$ (carbon isotope composition). It is calculated from $\delta^{13}C$ of the plant tissue, and $\delta^{13}C$ of the air (usually a constant).

Four levels of irrigation were employed and soil water content monitored. Above-ground biomass was determined for two early growth stages ("young plants") and for the mature crop, and this was compared to marketable yield. In addition, carbon ($\delta^{13}C$) and oxygen ($\delta^{18}O$) isotope composition were determined, and in some cases additional data for WUE_i were collected using IRGA. It was desirable to establish whether the biomass response to irrigation in young plants could be used as a convenient estimate of the biomass response of mature plants and therefore of an economically meaningful measure of water-use efficiency at the field level.

4.1.2. Results and discussion
The four irrigation regimes at Gleadthorpe (sandy soil) produced progressive effects on biomass accumulation, $\delta^{13}C$, $\delta^{18}O$ and WUE_i (see appended report for data). From these assays it appears that all genotypes responded to reduced irrigation by increasing WUE, and by reducing transpiration and biomass, as expected. Significant genotype (G) and irrigation (I) effects were found for biomass, $\delta^{13}C$ and $\delta^{18}O$, and G x I effects were apparent for $\delta^{13}C$. The two Nedcha x Gower lines that showed extreme $\delta^{13}C$ values in the earlier glasshouse trial [6] (NG132a, high WUE; NG142, low WUE) showed the same behaviour in our two field trials. The two extreme irrigation treatments at Gleadthorpe were found to be suitable for future trials of mapping populations and diversity sets.
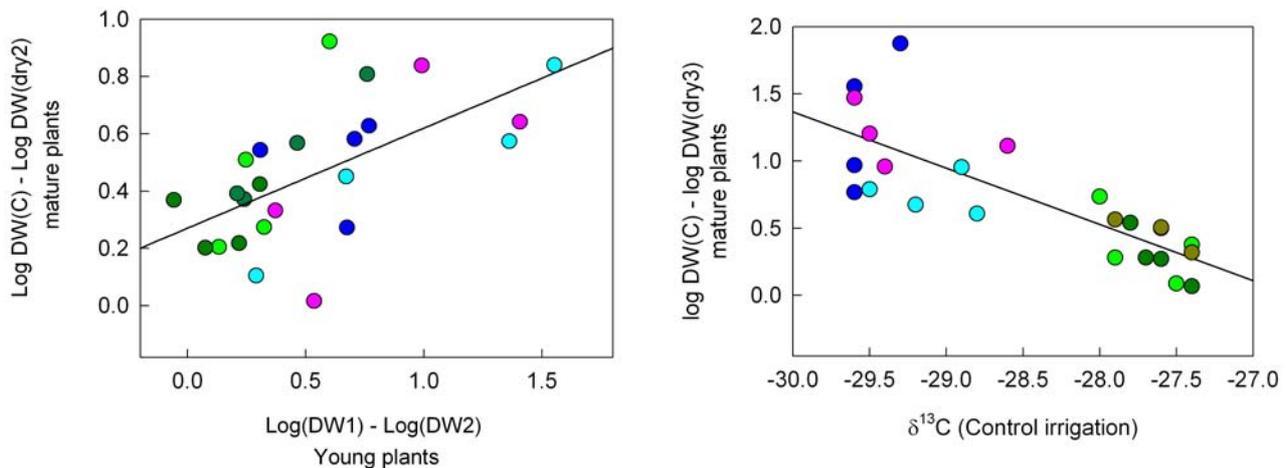
Figure 6:Left panel: Relationship between the dry weight response to irrigation in young plants compared to mature plants. The differences in log plant DW (arbitrary units) at two levels of irrigation (1 and 2) are shown. Different symbol colours represent different genotypes. **Right panel: Negative relationship between $\delta^{13}C$ and biomass response to irrigation.** Each colour is a different genotype. Different shades of green are the cabbages.

At the Kirton site (with a heavier soil) no reduction in growth was detected even in covered crops that had been irrigated at transplantation and then received no further irrigation during the season. It is likely that these unirrigated plants at Kirton did not experience such severe soil water deficits as produced at Gleadthorpe due to the superior water holding capacity of the soil and the higher level of ground water. Isotope analyses were performed on the two extreme irrigation treatments at Kirton, and it was found that despite the lack of growth effects, there was a significant improvement in WUE_i in the non-irrigated crop as measured both by $\delta^{13}C$ and IRGA. This suggests that the plants were responding to signals from drying upper layers of soil, but that the response was such that while it limited excessive water loss the stomatal closure was insufficient to limit assimilation. **This is an important finding as, similar to the findings with potato described above, it shows that drying soil leads to an increase in brassica WUE without an accompanying loss in crop yield, and illustrates the potential to genetically improve, or agronomically manipulate, WUE without yield loss.** From this experience, growing protocols for subsequent trials at Kirton were modified in order to reduce soil water content prior to transplantation so that a growth response to water deficit may be achieved.

We generally found that the biomass response of young plants to irrigation was reflected in the response of the final crop yield, particularly when the response of each genotype is considered separately (Figure 6). We concluded that measurements on young plants could be used as a reasonable estimate of mature plants, and so can be used for further QTL and diversity trials.

Further analysis of the Gleadthorpe data showed that the response of biomass to irrigation at the final harvest was correlated with the leaf level indicator of WUE, $\delta^{13}C$, in irrigated plants (Figure 6), although this relationship was dominated by the crop type (cabbage vs non-cabbage). Thus genotypes whose yield was least affected by water deficit also had the greatest WUE as measured by $\delta^{13}C$, and tended to be cabbages rather than other types of vegetable brassica.
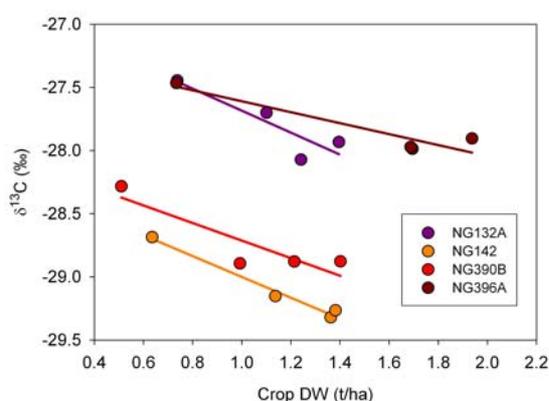

Figure 7: Relationship between yield and WUE for selected N x G doubled haploid lines. For each genotype the mean of replicates is shown for each of the four irrigation treatments.

The Gleadthorpe data also indicated two different types of relationships between WUE and yield: driven by plant response to water deficit, or driven by genotype. It was apparent that each genotype responded to water deficit by reducing transpiration and biomass, and increasing WUE, and thus for each genotype there was a negative relationship between yield and WUE caused by environmental response (e.g. Figure 7). Note that this contrasts with the situation a Kirton where a modest increase in WUE and drop in transpiration in the unirrigated treatment was not converted into a yield decrease, as discussed above. However, when all treatment and replicate data were averaged for each genotype, to give an integrated measure of genotype performance under a range of soil water potentials it was clear that high yield was not always associated with either low WUE or high transpiration (Figure 8). For example, genotypes NG396A and NG132A both had similar high WUE based on $\delta^{13}C$, but NG396A

had a higher yield in irrigated treatments (Figure 7). Similarly, in the case of commercial cultivars, "Attraction" had high WUE, low transpiration and high yield, whereas "Fremont" had low yield, low WUE and high transpiration (Figure 8). Thus the negative relationship between yield and WUE caused by environmental response was not upheld between genotypes. This suggests that some genotypes may have higher WUE as a result of higher assimilation rather than simply through decreased transpiration. **This is an important finding as it indicates that it is possible to select for genotypes with both high yield and high WUE, and that a combination of $\delta^{13}C$, $\delta^{18}O$ and biomass data can be used for this selection process.**
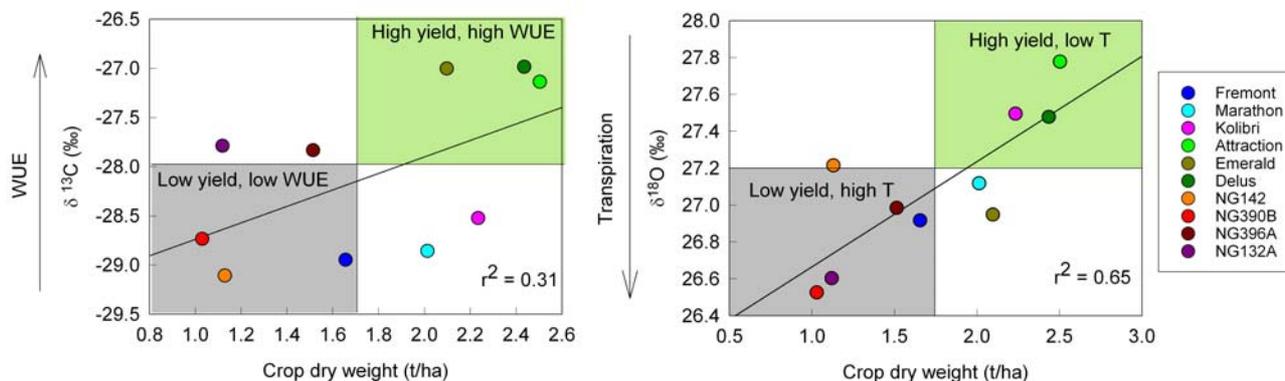

Figure 8: Selection of high yielding, high WUE genotypes using biomass, $\delta^{13}C$ and $\delta^{18}O$. Data for 10 genotypes, young plant stage at Gleadthorpe. Each point is the mean of 4 treatments x 4 reps (n=16). Plots are divided into quadrants to demonstrate a possible genotype selection approach. Green quadrant is desirable, grey undesirable. T = transpiration estimated by $\delta^{18}O$. Arrows indicate the direction in which WUE or transpiration increases (according to theory, $\delta^{18}O$ is negatively correlated with transpiration).

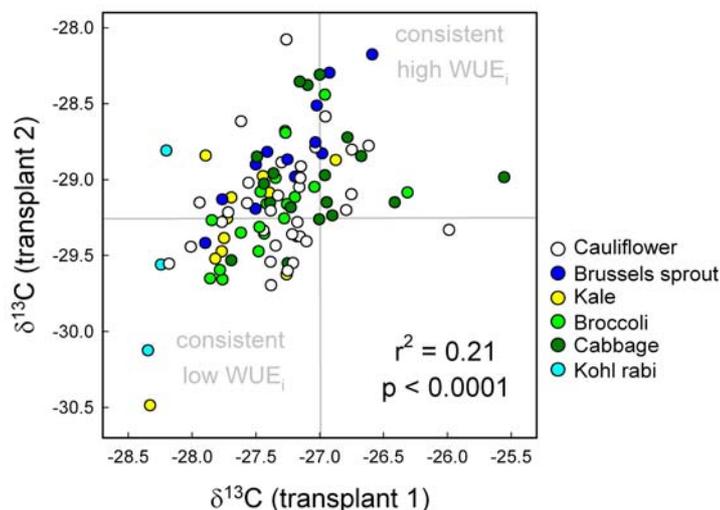**Figure 9: Genetic diversity of $d^{13}C$ in the *Brassica oleracea* diversity set.** Each point is a genotype mean of 6 plot replicates (combining control and drought data) for each of two transplantings. Data from the two consecutive transplantings are plotted against each other to identify genotypes with robust high or low WUE_i.

4.1.3. Conclusions – Brassica method development

• Response of biomass accumulation to irrigation in young *B. oleracea* plants can be used as a reasonable economically meaningful measure of water use efficiency at the field level as it is likely to reflect response of final yield.

• WUE_i (from $\delta^{13}C$ data) and yield decreased in response to water deficit, as expected, but the relationship between yield and $\delta^{13}C$ when comparing genotypes was weak, suggesting that these two traits were under independent genetic control.

• Extreme $\delta^{13}C$ values in doubled haploid NxG lines were reproducible between the previous glasshouse trial (Hall) and our field trial, suggesting good heritability of the trait.

• We have illustrated in principle how combination of $\delta^{13}C$ and $\delta^{18}O$ and biomass values can be used to select genotypes for high yield and high WUE.

4.2. Objective 02: Assessment of genetic diversity of WUE (Brassica)

A set of *B. oleracea* accessions were selected by colleagues at Warwick HRI to represent the full range of phylogenetic diversity. These accessions are being converted into doubled haploid lines to create *B. oleracea* diversity fixed foundation set (BoIDFFS) lines. A subset of 107 BoDFFS founder lines, with sufficient seed availability, was grown in highly replicated field trials under Spanish tunnels at Kirton in 2006. Two plantings took place, 20 June and 16 August, and on each occasion there were 642 plots of 8 plants representing all combinations of lines under two irrigation regimes (control and dry) replicated three times. During growth, targeted IRGA measurements were taken, focusing on the broccoli crop type. After approximately six weeks, the final harvest was made and measurements of fresh and dry weight, specific leaf area (SLA) and leaf chlorophyll content (SPAD) were taken. Stable isotope ($\delta^{13}C$ and $\delta^{18}O$) and mineral analysis was also performed on leaf samples (for project HH3501SFV). Data sets from these BoIDFFS

field trials were used in combination with genotype allele data to perform an allelic association analysis in an attempt to validate and resolve QTL (see objective 4, section 4.4).

As expected in this diverse collection of genotypes, we observed significant treatment and genotype effects for all traits measured, and in many cases treatment x genotype interactions were also detected. The range of WUE_i values, as measures by $\delta^{13}C$ is illustrated in Figure 9. For example, for Kales we observed a range of -30.5 to -28.8 ‰; extrapolating from data with tomato genotypes, this represents a difference in WUE of about 30% [7].

4.3.   Objective 03: Identification and resolution of robust WUE QTLs (Brassica)

Identification of water-use efficiency QTLs in *B. oleracea* focused upon the doubled-haploid A12DH x GDDH33 (AxG) mapping population from a cross Chinese Kale x broccoli. A series of highly replicated field trials were carried out in 2005 in order to provide robust phenotype data for the QTL analysis. The level of replication required dictated that our QTL analysis focused on a subset of 84 lines from this population which had sufficient seed availability.
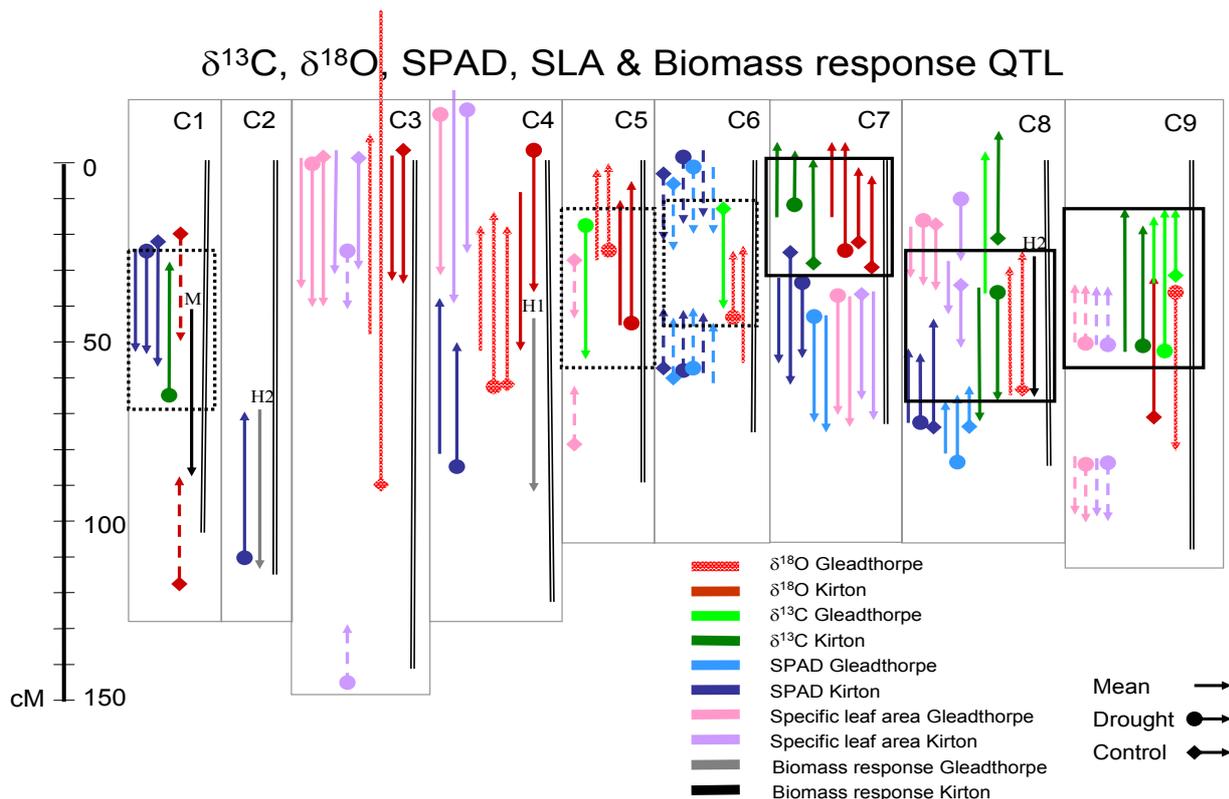

Figure 10: Composite A12 x GD33 DH QTL map for all traits analysed. QTL are displayed individually for each treatment (drought & control, depicted by the symbols at the line ends) at each site in addition to the mean QTL for each site. Biomass response QTL are annotated M=mean, H1=harvest 1 & H2=harvest 2. C1 to C9 represent the *B. oleracea* linkage groups. Three regions of the genome with the most compelling QTL evidence for involvement with WUE are highlighted on LG C7, C8 and C9 (black rectangles, solid lines). A further three regions where the evidence is less strong (LG C1, C5 and C6) are also indicated (black rectangles, dotted lines). An up arrow indicates a positive effect on the trait by the A12 parent. A down arrow indicates a positive effect on the trait by the GD33 parent.

4.3.1.   Design of field trials for QTL analysis in the AxG population

Two sequential transplantings were made at each of the two contrasting sites. At Kirton, irrigation was by trickle, the crop was fully covered under Spanish tunnels. Gleadthorpe was irrigated by overhead sprinkler boom, and dry plots were protected from rainfall by mobile rain shelters. At Kirton irrigation treatments were; CONTROL = 20 mm at 25 mm soil moisture deficit (SMD); DRY = no irrigation. At Gleadthorpe, control treatments were rain-fed and received an additional 20 mm at 25 mm SMD. Dry treatments received 50% of the control treatment volume. Treatment differences in SMD were achieved as recorded by Diviner 2000 readings. Transplanting 1 was harvested after 6 weeks, and it was observed that some of the genotypes had begun to flower at this stage. Transplanting 2 was grown for less time such that the majority of lines had not yet begun to flower. It is anticipated an interaction between flowering and WUE may confound the QTL for WUE so it is desirable to harvest the plants prior to flowering. The following data were collected from the crops for assessment of WUE: leaf gas exchange, fresh and dry weight at harvest, mineral and stable isotope analysis, leaf chlorophyll content, specific leaf area (cm^2/g DW).

4.3.2. QTL analysis

QTL analysis was performed using the above trait data in combination with raw genotyping scores for robust markers mapped on the AxG mapping population. The QTL analysis was carried out using a subset of 88 loci evenly spaced at approximately 10 cM intervals with a marker regression approach based on line means [8] using the QTL Café application and then verified with WinQTL Cartographer v2.5. In general, data at Gleadthorpe were more variable than at Kirton, and more significant QTL were observed at Kirton. There was greater residual variation in biomass data than for other traits, and in some cases there were differences in the QTL detected between transplantings and sites. However, several QTL were found to be consistent between transplantings and sites, e.g. specific leaf area on C3 and δ^{13}C on C9 (C3 etc, indicates linkage group 3 in the brassica "C" genome). The significant ($P < 0.05$) WUE$_i$ QTL we have identified are on linkage groups C1, C5, C6, C7, C8 & C9; of these C7, C8 & C9 are the most consistent because they have been repeatable across sites, treatments and/or transplantings. When selecting the genomic regions on which to focus further investigation, consideration was also given to the δ^{13}C QTL previously identified in the NxG population on C7 and C8.
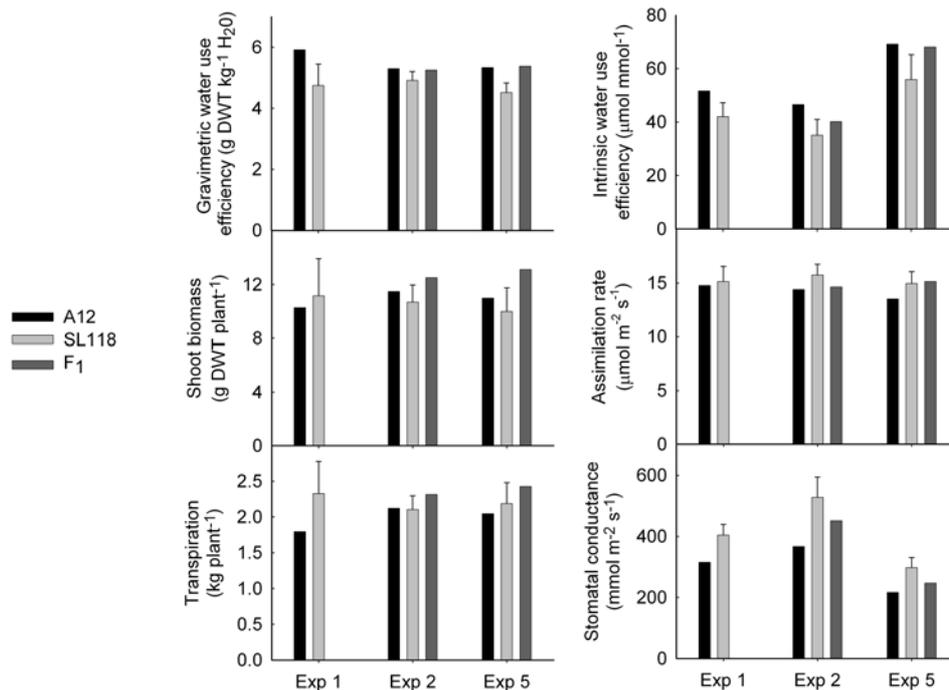

Figure 11: WUE of substitution line SL118 in three glasshouse experiments. Plants were grown in pots, in a partially environmentally controlled glasshouse and SL118 was compared to the recurrent parent A12 and the F$_1$ hybrid. Stomatal conductance (g_s) and CO$_2$ assimilation (A) were measured by infrared gas analyser (IRGA), and A/g_s (intrinsic water use efficiency) was calculated. Pot weight was used to determine water lost by transpiration, and shoot dry weight (DWT) was determined at the end of each experiment. Gravimetric WUE was then calculated (biomass gain/total plant transpiration). LSD (5%) is given as an error bar arbitrarily on the SL118 data. This is for comparisons between A12 and either SL118 or the F$_1$ within each experiment. Between 6 and 10 replicate plants were used per line.

4.3.3. Substitution lines to test WUE QTL in the NxG map

In addition to the AxG mapping population, the 2005 field trial also contained 14 selected _Brassica oleracea_ AxG substitution lines (BoAGSL) that contain introgressions of GD33 in an A12 background [9]. Introgressions were selected to coincide with WUE QTL previously identified in the Nedcha x Gower mapping population under both field and glass conditions [5]. For these lines, significant differences in comparison to the recurrent parent (A12) were found at Kirton for specific leaf area and SPAD, and at both sites for δ^{13}C and δ^{18}O (tabulated in the appended report). Nine lines had significantly different δ^{13}C, and three of those were consistent between sites. This data provides additional evidence for QTL for these traits in the substituted regions.

Subsequent to the identification of QTL in the AxG population a series of highly replicated field trials comprising selected AxG substitution lines was carried out in 2007: 24 and 28 BoAGSL lines, and the A12 parent, were grown at two levels of irrigation at Gleadthorpe and Wellesbourne respectively. This provides an opportunity to validate and improve resolution of the AxG QTL by fine mapping of substitution lines that show significantly different trait values.

4.3.4. Investigation of substitution line SL118 in the glasshouse

The most significant BoAGSL with substitutions in the region of QTL detected in the AxG population were investigated in a series of glasshouse experiments. Line SL118 gave the most consistent evidence for a difference in WUE: A12 and SL118 were compared in three separate experiments, and both gravimetric whole plant WUE (WUE$_p$) and intrinsic leaf-level WUE (WUE$_i$) were significantly lower in SL118 in all three experiments

(Figure 11). This is in agreement with $\delta^{13}C$ data collected in the field, where SL118 had more negative values (indicating lower WUE) in several trials, and in agreement with the QTL found at the top of linkage group 7, where A12 provided the increasing allele for $\delta^{13}C$. **Typically A12 had 15-20% higher WUE than SL118, and up to 25% lower g_s.**

SL118 was included in the field trial because of it's introgression on linkage group 7 in the region of a QTL for $\delta^{13}C$ (Figure 10), but it also contains further introgressions on linkage groups 1 and 6. For this reason we have produced a BC_1F_2 seed stock for the cross A12 x SL118, with the aim of isolating and reducing the size of the introgression on linkage group 7. Screening this seed stock for lines that have lost secondary introgressions and recombined to reduce the size of the primary introgression is now being completed as part of a new Defra follow-up project.

We investigated four other substitution lines in the glasshouse (SL169, SL160, SL155 & SL165). Of these only SL160 appeared interesting as it had lower g_s than A12 in many field and glasshouse experiments, and also because it had a significant increase in the ratio of abaxial to adaxial g_s in the glasshouse experiments (in this case the F_1 indicated the SL160 parent provided the dominate allele), suggesting genuine differences in stomatal distribution and/or behaviour. However, there was no evidence that this had an effect on WUE at the whole plant level, and, although we did also generate BC_1F_2 seed from this line, further investigation of this line was considered to be a lower priority than SL118.

4.3.5. Objective 03: conclusions
- Robust QTL for $\delta^{13}C$ (surrogate measure of WUE) and $\delta^{18}O$ (surrogate measure of leaf transpiration) have been established in the AxG population.
- Regions of linkage groups C7, C8 & C9 have been highlighted as the most worthy of further investigation because they have the strongest QTL effects and have demonstrated repeatability across sites, treatments, transplantings and mapping populations.
- In a region of linkage group C7, evidence from stable isotope analysis points to a QTL where high WUE and low transpiration are associated with a chromosomal region from the A12 parent. Substitution line SL118, with a C7 introgression, strongly confirms this QTL.

4.4. Objective 04: Validation of QTLs by association mapping
The QTL maps described above are derived from a single mapping population, AxG, with supporting data from the Nedcha x Gower population, and thus a maximum of four alleles at each locus were assessed (one from each of the four parents). Allelic association analysis provides a means to assess a much larger "allelic space" and to look for associations between extreme trait values and particular genetic loci. If such loci can be found within the QTL described above then it would (i) confirm that these regions are of interest in breeding for WUE, (ii) potentially increase the resolution of the QTL map position and (iii) highlight particular alleles or haplotypes that would have beneficial effects in a breeding program.

In order to perform allelic association analysis, phenotypic data and locus specific genotyping data are required for a set of diverse germplasm. This project generated WUE trait data for the 107 *B. oleracea* diversity foundation set (BolDFFS) lines in field trials in 2006 (section 4.2).
Having identified and prioritised the most interesting *B. oleracea* WUE QTL regions (Objective 03), additional single sequence repeats (SSR markers) were identified in these regions (see appended report), and the 107 BolDFFS lines were genotyped with these markers. Also, genotyping data for 100 markers distributed around the genome for a 'core set' of 96 BolDFFS lines was available (Guy Barker and Dr Gongjun Shi, Warwick HRI) and 32 lines from this core set are in common with the 107 WUE BolDFFS lines we grew in our field trial. Genotype scores for these 32 lines were included in our allelic association analysis thus bringing the number of *B. oleracea* markers in our analysis to 109.

The trait data were analysed using REML to calculate the effect of line, treatment and the interaction between the two. Line means for each trait, together with line means for each treatment where the line by treatment interaction was significant, were then compared with the markers in a linkage disequilibrium association analysis. For each trait, a t-test was performed to detect association between the line means and the presence or absence of each allele of all the 109 markers. The significance of the association between alleles and a trait was assessed using the Benjamini Hochberg test, with a false discovery rate (FDR) of 0.05 (Figure 12).
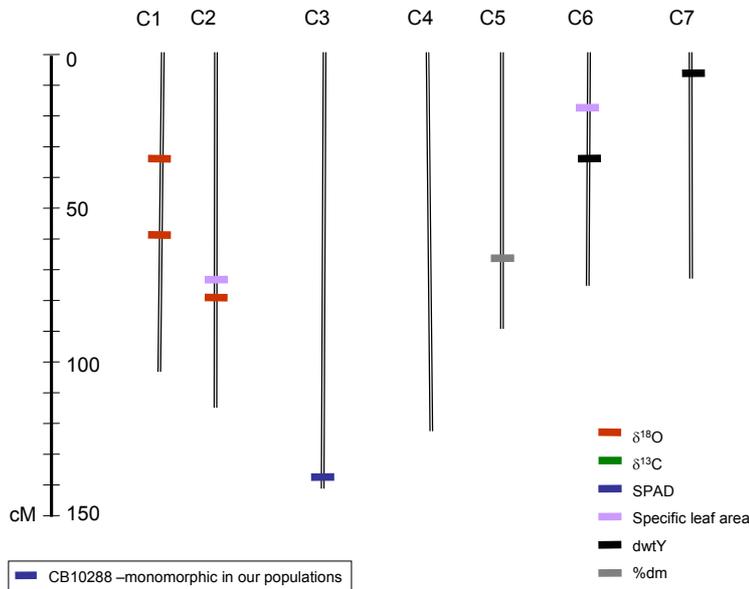
Figure 12: Physical representation of the significant allelic associations detected in *B. oleracea*. Note that some markers have more than one allele associated with the same trait. Also, some marker alleles are associated with the mean and individual treatment values of the same trait eg. BN35DR1_223 (C8, 9cM for δ^{18}O and δ^{18}OD. Marker CB10288 associated with SPAD, but it could not be mapped because it was not polymorphic between A12 and GD33.

When overlaying the results of the QTL analysis with these allelic association results, two areas of agreement are apparent: the mid regions of C8 and C9. On C8 there are four allelic associations collocating with QTL, two with δ^{18}O, one with dry weight yield and one with SPAD. On C9 there are five significant associations collocating with QTL, three with specific leaf area, one with δ^{18}O and the sole association found for δ^{13}C (Figure 13).
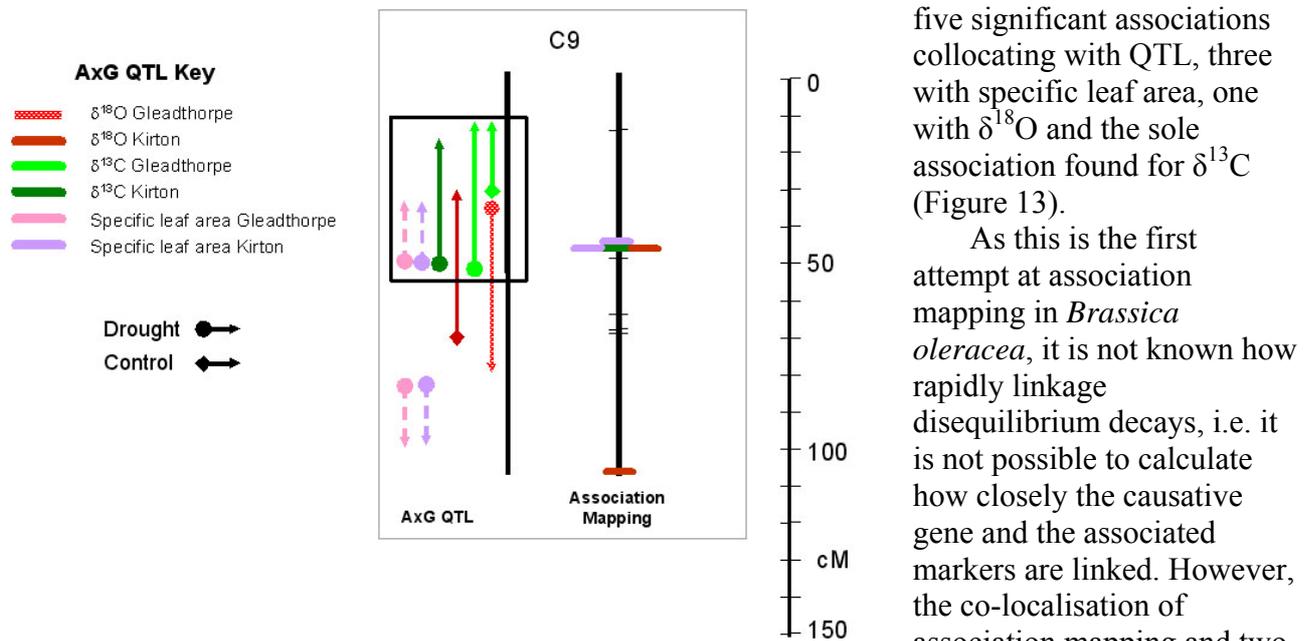

Figure 13: Comparison of QTL on C9 identified in either the AxG population or by association mapping. AxG QTL are as described in Figure 10, and association mapping QTL are described in Figure 12. SLA, δ^{13}C and δ^{18}O colocalise using the two different techniques.

As this is the first attempt at association mapping in *Brassica oleracea*, it is not known how rapidly linkage disequilibrium decays, i.e. it is not possible to calculate how closely the causative gene and the associated markers are linked. However, the co-localisation of association mapping and two-parent mapping QTL for δ^{13}C on C9 strongly suggest that this region should be targeted by further research and as a potential breeding target.

4.4.1. The magnitude of QTL effects

On C9, the locus associated with a difference in the δ^{13}C trait was THL-SSR-1; when comparing the mean of lines with and without the 149 bp allele of this marker, the presence of this marker was associated with an increase in δ^{13}C of 0.29‰. Similarly, in the AxG mapping population the A12 haplotype at this locus had a mean increasing effect of 0.27‰ on δ^{13}C compared to the GD33 haplotype. We can estimate very approximately the effect of this on the more tangible measures of WUE by using the plots of δ^{13}C against WUE$_p$ (gravimetric whole plant WUE, Figure 4, *Arabidopsis*) and A/g_s (instantaneous, intrinsic WUE, Figure 1, potato): an increase in δ^{13}C of 0.29‰

represents in increase of 0.183 mg DW g^{-1} in gravimetric WUE (for the average *Arabidopsis* accession this would be an ~4% increase), or an 0.006 mol mmol^{-1} increase in A/g_s (about an 8% increase for an average potato genotype).

The size of the C7 QTL effect on δ^{13}C calculated from the AxG population data was smaller, at only 0.17‰, equivalent to an estimated 3% change in WUE$_p$. However, direct measurement of WUE$_p$ in the substitution line SL118, where the GD33 introgression at the C7 QTL was isolated in a mainly A12 background, indicated a much larger mean effect of 17%, at least in glasshouse conditions (Figure 11). By comparison, the difference between the maximum and minimum genotype mean for δ^{13}C within the entire Brassica oleracea diversity set was 2.11‰, which we estimate to represent a difference of about 30% in WUE$_p$. **Thus the 17% difference in WUE$_p$ between A12 and SL118 is a relatively large QTL effect with potential utility in breeding programs.**

5.   Comparative genetic and genomic anaysis (*Brassica-Arabidopsis*)

We have attempted to localize the *Brassica oleracea* QTL regions on the *Arabidopsis* genome, and vice versa. However, the large size of the mapping intervals means that it is not possible with any confidence to say that a QTL detected in *Arabidopsis* can be mapped to a particular region of *B. oleracea* (or vice versa), mainly because the extent of synteny (i.e. the phenomenon whereby genes are in the same order on the chromosomes of different species) operates only at the scale of a few cM. When comparing *B. oleracea* and *Arabidopsis*, typically a section of DNA of 5 cM in size would map to several regions of the other genome because of the large number of translocations and inversions that have taken place during evolution, giving a complex mosaic of microsyntenous regions. This is further complicated since the *B. oleracea* genome is a triplicated version of the *Arabidopsis* genome (which has itself undergone duplication). We anticipate that if QTL can be localized to less than 1 or 2 cM, then it would be possible to transfer this information between species. The association mapping hits in *Arabidopsis* typically would represent about 100 kbp to 200 kbp, and this sequence could be used to easily find syntenic loci in *B. oleracea* when the genomic sequence becomes available. Thus, in this project it has not been possible to make useful comparative analyses, but we have initiated projects to continue to fine map QTL in both *Arabidopsis* and *B. oleracea*, and to perform more association analysis. The more detailed mapping information obtained, together with strengthening genomic resources, will allow comparative analysis in the future.

We have looked for likely candidate genes in the locations of association mapping hits in *Arabidopsis* where they co-map with *Arabidopsis* WUE QTL. This procedure would typically generate a list of 20-30 genes per hit, and it is then possible to perform literature searches and to form hypotheses regarding the possible involvement of specific genes in a particular trait. For example, for the Arabidopsis QTL at the top of chr4 we scanned the genes surrounding an association hit within this QTL using At-ensembl genome browser (http://atensembl.arabidopsis.info/index.html), and were able to identify at least three genes with functions that might relate to WUE: an ABA responsive putative ABA binding protein (At4g01600), an aquaporin (At4g01470) and an expansin (At4g01630). Association mapping with increased density of genetic markers, construction and phenotyping of NILs, and transgenics can be used to investigate such candidate genes.

6.   Overall Conclusions

01: Development of methodologies for measurement of WUE

- Where we were able to measure WUE using different methods (δ^{13}C, A/g_s and WUE$_p$) in the same material, the methods were in general agreement, supporting the view that δ^{13}C can be relied on as an integrative, high-throughput measure of WUE in the field.
- We have defined field conditions and irrigation treatments that are suitable for detecting genotype differences in WUE for *Brassica* and potatoes as measured by δ^{13}C and IRGA.
- We have demonstrated how the use of a combination of δ^{13}C, δ^{18}O and biomass data could be used to select genotypes with high WUE while selecting also for high yield potential.
- We have shown that δ^{13}C is a highly sensitive measure of crop response to soil water deficit, and based on this we have also proposed that selecting genotypes whose δ^{13}C shows a minimal response to imposed soil water deficits is an attractive alternative strategy to select genotypes that avoid water stress (e.g. by foraging for water at depth).
- In several field experiments we observe that mild water deficits, through physiological responses, increase WUE with little effect on yield. This supports the idea that high WUE and high yield are not mutually exclusive, and that breeding for these combined traits is an attainable goal.

02: Assessment of genetic diversity of WUE

- We have described the genetic diversity in WUE, and related traits, in three species: in potato genotype means for δ^{13}C across 20 genotypes fell in the range -27.75 to -25.9 ‰. For 107 *Brassica oleracea* genotypes this range was -28.3 to -25.5 ‰. For 96 *Arabidopsis* genotypes this range was -31.9 to -29.5 ‰, and the most efficient genotype had 80% greater WUE than the least efficient genotype, as measured by dry weight gain per unit of water transpired.

- Genetic manipulation to increase ABA content in Arabidopsis genotype Col-0 led to a 56% improvement in WUE, and the maximum WUE achieved by this method was greater than that of any natural accession.

03: Identification and resolution of robust WUE QTLs

- We have identified five genetic loci in *Arabidopsis* that control WUE; four of these are supported by both and gravimetric (WUE_p) data, and three are not previously reported.
- In *Brassica oleracea* we have identified six QTL for, and we have highlighted three regions (C7, C8, C9) where QTL are reproducible between transplantings or sites.
- In addition to $\delta^{13}C$ data, we have collected biomass, $\delta^{18}O$, SPAD and specific leaf weight data (plus WUE_p for *Arabidopsis*). Co-localising QTL for these traits provides clues about the physiological causes of the $\delta^{13}C$ QTL.

04: Validation of QTLs

- In *Arabidopsis,* we have begun to construct near isogenic lines in the region of QTL detected in the Col-gl1 x Kas-1 population. We have data supporting the existence of two WUE_p QTL after two backcrosses.
- In *Arabidopsis,* we have used association mapping in 96 accessions to detect loci within the Col-gl1 x Kas-1 QTL that are associated with WUE_p and $\delta^{13}C$; on this basis we have highlighted genes close to those markers that might be causative.
- In *Brassica oleracea* we have highlighted a region of C7 that controls WUE based on both A12 x GD33 and Nedcha x Gower mapping populations. We have validated this by detecting the QTL in substitution lines in field and glass experiments. Physiological assessment of substitution line SL118 shows 17% effects on g DW kg^{-1} H$_2$0, driven by changes in stomatal conductance.
- The QTL on C9 detected in the A12 x GD33 *Brassica* population was validated by performing association analysis; we discovered that a marker within this QTL that was associated with $\delta^{13}C$.

Application of QTL for WUE in plant breeding

- Our genetic analysis could be applied to breeding for WUE provided that further fine mapping of QTL can be achieved so that tightly linked markers can be offered to breeding companies. Such markers could be used for MAS to ensure that beneficial alleles for WUE were maintained in breeding programs while phenotypic selections for unrelated traits were made. This would raise the baseline for WUE in breeding populations. By combining markers for several key WUE QTL, incremental increases in WUE should be achievable.

7.   Future work

We are fine mapping Arabidopsis QTL for WUE in project WU0116 by backcrossing and screening for recombinants, and we are developing improvements in genome-wide association mapping in *Arabidopsis* (where 240,000 single nucleotide polymorphism (SNP) loci have been recently made publicly available for such studies). Once the underlying genes are identified in Arabidopsis, we can test their utility in field crops.

We are fine mapping the QTL for WUE on C7 in *Brassica oleracea* by the same approach. This report contains the first attempts at association mapping in *Brassica oleracea* and the strong QTL for $\delta^{13}C$ on chromosome C9 was confirmed by our association mapping data. This provides a target region that would be ideal for more refined mapping and then allele testing. In the future we will need to increase the density of molecular markers in the target regions by employing high throughput SNP platforms; this will allow fine mapping of QTL and is required to find more closely linked markers suitable for marker-assisted selection (MAS).

Data from this project could be applied to other *Brassica* crops, particularly broad acre oilseed crops where WUE and drought tolerance are of great interest globally. For example, once fine mapping of QTL is more advanced, we could perform targeted association analysis, and construct near-isogenic lines in *Brassica napus* for testing in the field.

Our work in transgenic tomato has shown that single transgenes can have dramatic effects on WUE_p that can exceed the natural variation available (e.g. 80% increase compared to 17% increase for the best *Brassica* QTL), and that have little or no impact on biomass production [7]. This approach should be pursued in parallel to the exploitation of natural variation to make the most rapid advances in the breeding of high WUE crops.

In potato there is an urgent need to develop cultivars that can be productive with reduced irrigation. We believe it is timely to consider screening approaches using physiological rather than genetic markers in this species. In section 2.2.2 we have suggested ways of selecting with $\delta^{13}C$.

8.   Technology transfer (Objective 05):

8.1. Technology transfer events: interactions with growers and plant breeders

- An event, primarily for growers, was held at Kirton on 28th July 2004 entitled: "Herbicides, irrigation and nutrition: an update on research on field vegetables and outdoor cut-flowers". At the event talks were given by Dr Gordon Hanks who described the aims of the project.
- Representatives of the seed industry attended a "Seed Industry Day" at Wellesbourne on 12th April 2005. Dr Andrew Thompson presented a project overview entitled "Genetic control of traits related to water use in horticultural crops".

- An open day was held at Kirton on 23rd August 2005 and a poster describing this project was presented to growers and members of the public. "Breeding for Improved water-use efficiency."
- Warwick HRI Water Day. 20th July 2006: Andrew Thompson gave a presentation about this project in a talk entitled "Breeding for improved water use efficiency".
- Discussions were held with Syngenta seeds at a meeting at Wellesbourne in March 2008.
- Results were presented by Prof. David Pink to Plantum, a Dutch plant breeding organization, at the Brassica 2008 meeting in Lillehammer, Norway. Sept 2008.
- The *B. oleracea* data generated by this project will be included on the 'CropStore' database (http://www.brassica.info/ukbrc/advab/).

8.2. Articles in the popular media and peer reviewed publications

Three popular articles and one iCAST have been produced during the course of the project and are listed in the SID5 section 9. Publications for peer review in preparation are also described in section 9.

8.3. Scientific oral presentations

UK-Brassica Research Community meeting, March 9th, 2004, Warwick-HRI, Wellesbourne, "Genetic control of water use efficiency", Dr Andrew Thompson.

SCRI, Invergowrie, Dundee. March 3rd 2005 "Genetic control of traits related to water use in horticultural crops", Dr Andrew Thompson.

UK-Canada Brassica Genomics Workshop, October 2006, Brocket Hall, Herts. Identification of genetic markers for water-use-efficiency (WUE) in horticultural crops. Dr Andrew Thompson.

UK-Brassica Research Community meeting. Rothamsted, UK. (2007) Identification of genetic markers for water-use-efficiency (WUE) in horticultural crops. Dr Andrew Thompson.

University of Westminster, Department of Biological Sciences Seminar Series, London, UK. (2007) Genetic control of water use efficiency in crop species. Dr Andrew Thompson.

Invited Seminar, SCRI, Dundee. (2008) Improving water use efficiency in field crops. Dr Andrew Thompson.

Brassica2008, Lillehammer, Norway. (Sept. 8th-12th, 2008) Selected presentation for trait genetics session. "Water-Use-Efficiency Genes in *Brassica oleracea*" Carol Ryder

Jan 2009: AJT Invited speaker: Understanding and Exploiting Plant Signalling: A JXB Meeting to celebrate the contributions to plant science made by Ernst Steudle and Wolfram Hartung, Jan. 2009

March 2009: AJT Invited speaker: Academia Sinica, Taipei, Institute of Plant and Microbial Biology, Sensing, response and adaptation to altered water status.

8.4. Scientific posters

Deswarte, J.C., Hilton, H., Lynn, J., Hanks, G., Hughes, P., Teakle, G.R., White, P.J., Pink, D.A.C., King, G., Farquhar, G.D., Groves, S.J., Thompson, A.J. (2005) Genetic control of water use efficiency in Brassica and *Arabidopsis*. The 2nd International Conference on Integrated Approaches to Sustain and Improve Plant Production Under Drought Stress (Interdrought II), Rome September 2005.

Deswarte, J-C., et al. (2006) Genetic control of water-use efficiency in *Arabidopsis*. Water Day, Warwick HRI, 2006.

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6. Hall, N.M., *Stable isotopes, molecular markers and water use in Brassicas.* 2000, PhD Thesis, University of Newcastle: Newcastle upon Tyne. p. 1-181.
7. Thompson, A.J., et al., *Over-production of abscisic acid increases water-use efficiency and root hydraulic conductance and influences leaf expansion.* Plant Physiology, 2007. **143**: p. 1905-1917.
8. Kearsey, M.J. and V. Hyne, *QTL analysis - a simple marker-regression approach.* Theoretical and Applied Genetics, 1994. **89**: p. 698-702.
9. Rae, A.M., E.C. Howell, and M.J. Kearsey, *More QTL for flowering time revealed by substitution lines in Brassica oleracea.* Heredity, 1999. **83**: p. 586-596.

▮ References to published material ───────────

9. This section should be used to record links (hypertext links where possible) or references to other published material generated by, or relating to this project.

Publications generated by data from the project

1. **Deswarte, J-C** (2008) The genetic control of Water-Use Efficiency in *Arabidopsis thaliana* (L.) Heynh. PhD Thesis, University of Warwick.

Draft manuscripts

2. **Deswarte, J-C** (2009) Increasing water use efficiency beyond the range of natural genetic variation by tissue-specific LeNCED over-expression: a study of the impacts on growth and development. *Prepared for submission to Journal of Experimental Botany*
3. **Deswarte, J-C et al** (2009) New QTL for WUE discovered in Arabidopsis using complementary family-based linkage analysis and association mapping. *Prepared for submission to Plant Physiology or similar journal.*
4. **Ryder, C. et al** (2009) Mapping of QTL for water use efficiency and related traits in *Brassica oleracea*: use of RILs, substitution lines and association mapping. *In preparation.*
5. **Hilton, H. et al** (2009) Assessment of the water use efficiency of commercially available potato cultivars using stable isotopes of carbon and oxygen. *In preparation.*

Publications in related areas

1. **Tung S A, Smeeton R, White C A, Black C R, Taylor I B, Hilton H W, Thompson A J** (2008) 'Over-expression of LeNCED1 in tomato (Solanum lycopersicum L.) with the rbcS3C promoter allows recovery of lines that accumulate very high levels of abscisic acid and exhibit severe phenotypes.', *Plant Cell And Environment,* **31 968 - 981**
2. **Jones, M.O., Manning, K., Andrews, J., Wright, C., Taylor, I.B. and Thompson, A.J.** (2008) 'The promoter from SlREO, a highly-expressed, root-specific Solanum lycopersicum gene, directs expression to cortex of mature roots', *Functional Plant Biology,* **35** (12), **1224 - 1233**
3. **Thompson A J, Mulholland B J, Jackson A C, McKee J M T, Hilton H W, Symonds R C, Sonneveld T, Burbidge A, Stevenson P, Taylor I B** (2007) 'Regulation and manipulation of ABA biosynthesis in roots.', *Plant Cell And Environment,* **30** (1), **67 - 78**
4. **Thompson A J, Andrews J, Mulholland B J, McKee J M T, Hilton H W, Horridge J S, Farquhar G D, Smeeton R C, Smillie I R A, Black C R, Taylor I B** (2007) 'Over-production of abscisic acid in tomato increases water-use efficiency and root hydraulic conductivity and influences leaf expansion', *Plant Physiology,* **143** (4), **1905 - 1917**
5. **Taylor I B, Sonneveld T, Bugg T D H, Thompson A J** (2005) 'Regulation and manipulation of the biosynthesis of abscisic acid', *Journal Of Plant Growth Regulation,* **24 253 - 273**
6. **Thompson A J, Thorne E T, Burbidge A, Jackson A C, Sharp R E, Taylor I B** (2004) 'Complementation of notabilis, an abscisic acid-deficient mutant of tomato: importance of sequence context and utility of partial complementation', *Plant Cell And Environment,* **27** (4), **459 - 471**
7. **Thompson A.J.,** Tiffin, D. King, J. (2007) 'Opportunities for reducing water use in agriculture', Defra Project Report WU0101. http://randd.defra.gov.uk/Document.aspx?Document=WU0101_5888_FRA.doc

Articles in the popular media

1. **Thompson, A. J.** (2006) Breeding for improved water use efficiency in potato. *Vegetable Farmer* **June**: 25-27
2. **Thompson, A. J.** (2006) Water day at Warwick HRI *UK Irrigation* **34**: 34-36
3. **Thompson, A.J.** (2007) 'Climate change and its impact on water use in field crops', *Fresh Produce Journal* http://www2.warwick.ac.uk/fac/sci/whri/research/climatechange/cgwateruse/
4. **Thompson A. J., Hilton H.** (Feb., 2008) "Improving water efficiency in plants". **Warwick iCAST**. http://www2.warwick.ac.uk/newsandevents/icast/archive/s2week12/water/

Conference presentations and posters are listed in the main project report under the knowledge transfer section.