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Effect of abscisic acid applications on cold tolerance in chickpea (*Cicer arietinum* L.)

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ABSTRACT

A series of field experiments were undertaken at three locations in Khyber PukhtunKhwa (KPK) Province, Pakistan to assess the effects of low temperatures and phytohormone applications on chickpea (*Cicer arietinum* L.) growth and yield. These trials showed that ABA application (10^{-4} M) to 40-day-old plants (before the first seasonal frost) offset low temperature-induced growth and yield depression at harvest (200-day-old plants) by up to 17%. These yield improvements were mainly due to an increase in the number of seeds pod^{-1}. Growth room experiments were carried out under controlled environmental conditions to establish how foliar application of 10^{-4} M ABA to 40-day-old plants might improve seed production at harvest. The foliar application of 10^{-4} M ABA had no detectable effect on endogenous shoot or root ABA levels four-days after spraying or on biomass when plants were maintained in warm conditions. When exposed to night temperatures of −2 °C, however, the endogenous ABA levels increased dramatically in both control and ABA-treated plants, but this rise was more rapid after ABA application ($p < 0.01$); after 14 days, these plants had gained significantly more biomass than the unsprayed controls ($p < 0.05$). No evidence was found to suggest ABA affected the osmotic or water balance of plants, but parallel experiments have shown ABA reduced low temperature-induced cell damage. Analysis of the proteome of the shoot tissues of ABA treated and untreated plants by 2-Dimensional Gel Electrophoresis identified several proteins that are induced by low temperatures and/or by ABA application in chickpea and which may be involved in conferring cold tolerance. Attempts were made to establish the identity of these proteins using mass spectrometry but in all cases the results were ambiguous; a more complete protein data base for legumes is required before the function of these proteins can be inferred.

1. Introduction

Low temperature is one of the most important environmental constraints limiting the productivity and distribution of plants (Lang et al., 2005). In response to low, non-freezing temperatures many plants undergo a cold acclimation process, and the growth regulator abscisic acid (ABA) is believed to play an important role in mediating this response (Lang et al., 1994; Levitt, 1980).

The mechanisms by which ABA increases cold tolerance, however, are not well understood. Experiments with Arabidopsis ABA mutants suggested that some cold induced genes are activated through an ABA-dependent and some through an ABA-independent pathway (Yamazaki et al., 1995; Chinnusamy et al., 2004; Yamaguchi-Shinozaki and Shinozaki, 2005). Application of ABA to plants not previously exposed to acclimating temperatures confers a measure of chill resistance upon maize (Janowiak and Dorffling, 1996; Ristic et al., 1998; Janowiak et al., 2002), cucumber (Yamazaki et al., 1995), rice (Lee et al., 1997) and chickpea (Kumar et al., 2008). There is also evidence that exogenous ABA increases frost tolerance in brassicas (Lang et al., 1994; Tamminen et al., 2001). Studies at the biochemical and biophysical level have identified a number of changes that appear to accompany cold acclimation (Taulavuori et al., 2001). These include changes in the soluble carbohydrate content (Thakur et al., 2010), increases in the soluble proline levels (Lee et al., 1997), and subtle changes in the protein compliment (Xin and Li, 1992) and degree of fatty acid saturation in the plasma membrane of shoot cells (Bakht et al., 2006). Alterations in the composition of the plasma membrane are of particular interest as they may be responsible for suppressing osmotically driven expansion-induced cell lysis, the lethal process that occurs when non-acclimated cells are thawed (Steponkus et al., 1988; Uemura and Steponkus, 1999). The precise sequence

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of events, however, that links acclimating temperatures to changes in endogenous ABA levels, and then on to any of the observed responses that are reported to confer cold tolerance is not well established.

Gilmour et al. (2004) identified the CBF family of transcription factors that regulate a battery of *co*ld *r*esponse (*COR*) genes in the *Brassicaceae* (Jaglo-Ottosen et al., 1998; Cook et al., 2004; Gilmour et al., 2004). It is clear that the C-repeat Binding Factor (CBF) operate near the top of the signalling cascade as their transcription is elevated within 15 min from exposure to cold acclimating temperatures (Chinnusamy et al., 2003), and that Ca^{2+} (Knight et al., 1996) and protein phosphorylation signal cascades (Monroy et al., 1998) may be involved. Details are not clear, however, on the signalling events that operate downstream of the CBFs, nor precisely what biochemical and physiological mechanisms are directly under their control. There is a consensus, however, that ABA is not directly involved in this signalling pathway. Many ABA inducible genes contain a consensus 5′ cis-acting element in their promoters (ACGCGG/TC) and this has led to the characterization of the ABA Response Element (ABRE) regulon. In both cereals and Arabidopsis several protein factors are required to achieve full activation of the ABRE regulon but it appears it responds mainly to drought and salinity stress (Nakashima et al., 2009). None-the-less, there is considerable evidence for a central role for ABA in cold acclimation which must occur through the ABRE or other signalling pathways (Land and Palva, 1992; Thomashow, 1994; Nordin et al., 1993; Wang et al., 1995; Pruvot and Jacqueline, 1996; Capel et al., 1997; Tamminen et al., 2001; Rabbani et al., 2003). ABA is known to be a potent growth regulator but it has been reported to both suppress (Hose et al., 2000; Hartung et al., 2002; Wan et al., 2004) and stimulate (Sharp et al., 2000; Sharp, 2002; Sharp and LeNoble, 2002) vegetative and reproductive growth (Liu et al., 2010). ABA is also reported to affect reproduction in plants. Low endogenous levels of ABA are reported to correlate with increased grain filling in cereals (Yang et al., 2003, 2004), and application of low concentrations of ABA to developing grain stimulates grain filling (Ahmadi and Baker, 1999; Yang et al., 2001, 2003, 2004) but indirect effect through the shoot cannot be discounted. In contrast, others have reported no correlation between endogenous ABA levels and seed size in soybean (Schussler et al., 1984, 1991)

The legume chickpea (*Cicer arietinum* L.) is currently grown over the winter period (when water supply is adequate) in the nitrogen-depleted soils of Northern Pakistan. Its potential as a major crop in the region, however, is limited by its sensitivity to low temperatures, particularly frost, and consequently its range is restricted to warmer and more arid regions. Chilling temperatures during the reproductive phase reduces seed yield in chickpea. This arises through increased rates of flower abscission, and reduced gametophyte vigour (Clarke and Siddique, 2004; Nayyar et al., 2005a,b; Berger, 2007; Thakur et al., 2010; Berger et al., 2012). In these studies, flower abscission correlated well with high endogenous ABA levels. In an earlier study, we have shown that application of ABA to 40-day-old chickpea exposed to low temperatures altered the composition and biophysical properties of the plasma membrane, reduced cell lysis, and improved growth (Bakht et al., 2006). In this communication, we report on the effects of low temperature and ABA application on the growth and reproduction of field-grown chickpea plants (from seedling stage to harvest). Growth room experiments were also conducted to study the effect of ABA applications during the cold acclimation process (day 40–54). The aim of the present study was to establish whether increased endogenous levels of ABA in field-grown chickpea might improve vegetative growth and yield under cold conditions, and to determine which cold- and ABA-induced proteins may be involved in these responses. If improvements are observed it may be possible to boost yields by genetically enhancing endogenous ABA levels or the application of ABA to the crop.

2. Materials and methods

2.1. Field experiments

Field experiments were conducted on chickpea (*C. arietinum* L., cv. CM 72) at three locations that provided a gradient from warm to cool growth conditions. The ABA application experiments reported here were to assess the effects of temperature and ABA on chickpea growth and yield. The experimental factor Location was used as a proxy for temperature and henceforth will be referred to as Islamabad (warmest), SWAT1, and SWAT2 (coolest). Briefly, at each location three replicate blocks were set up, each containing three plots (6 m × 4 m). Three foliar ABA treatments were randomly assigned to each plot. At each location the soil was treated with fertilizer at sowing time (50/50/0 kg ha^{-1} P_2O_5/Urea/K_2SO_4). At all sites, chickpea seeds (cv. CM72) were inoculated with the *Rhizobium* strain SK-8 (both supplied by the National Agricultural Research Centre, Islamabad, Pakistan) for nodulation and planted in the first week of November and harvested in the third week of May. ABA treatment (aqueous solutions containing 0, 10^{-5} and 10^{-4} M ABA, Sigma A1049) was applied as foliar sprays (approximately 2 ml plant^{-1}) to plants 40 days after sowing, just before the onset of the first seasonal frost at SWAT2. At approximately 70 (first flower), 100, 150 and 200 (harvest) days after sowing, ten plants were randomly selected and harvested from each plot and shoot growth assessed (fresh weight, dry weight, and shoot length); in addition, at day 200 seed yield was estimated (pods/plant, seeds/pod and kg ha^{-1}). Recommended agronomic and irrigation practices were followed throughout the growing season, including control of weeds, and insect pests.

2.2. Growth room experiment

The growth room experiment was set up as a randomized split-plot design with ABA (2 levels; 0 and 10^{-4} M) and Time from Treatment (3 levels; 4, 7 and 14 days after low temperature and ABA application) as the main treatments; in total there were 180 plants in 36 pots (5 plants pot^{-1}). Growth room experiments were conducted at the University of Glasgow UK. Seeds of chickpea (*C. arietinum* L. cv. CM72) were soaked in distilled water overnight and five seeds were placed in 1 litre pots containing sand. The pots were kept at in a 20 °C/7 °C day/night temperature regime with a 12 h day/night photoperiod (450 μmol m^{-2} s^{-1}) and further randomized every second day to reduce positional effects. Plants were watered every week with 250 ml ½-strength Hoagland's solution. Forty-days after sowing, each pot was sprayed with either 10 ml of water (no treatment) or 10 ml × 10^{-4} M ABA, as above. The temperature in one growth room was then lowered to 5 °C/−2 °C day/night temperatures and the photoperiod maintained at 12 h (Cold treatment), whilst the temperature in a second growth room was maintained at 20 °C/7 °C day/night temperatures (control). Light was supplied both from fluorescent and incandescent bulbs. These conditions were chosen as they are similar to those encountered by 40- to 50-day-old plants in the field during winter at SWAT2. Plants were then harvested at 44, 47 and 54 days after sowing (representing 4, 7 and 14 days post treatment). Data were recorded for: shoot and root fresh and dry weight: shoot and root ABA content: shoot and root proline content: solute and water potential: in addition, total shoot protein was isolated for proteome comparisons by two dimensional gel electrophoresis.